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PHYSIOLOGICAL REVIEWS

VOL. VIII APRIL, 1928 No. 2

THE CONTROL OF POSTURE BY THE CENTRAL NERVOUS SYSTEM

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The walking reflex may best be studied when the act of walking is thought of as composed of two components, one static and the other dynamic. A tonic contraction of the extensor musculature counteracts the force of gravity and supports the body from the ground while alternate rhythmic movements of the extremities produce a change of position and are responsible for locomotion. "The execution of stepping movements by the limbs does not of course in itself amount to walking. For this latter act the reflex stepping of the limbs has to be combined with reflex maintenance of the erect posture of the body" (Sherrington, 1910).

Forbes (1912) and Graham Brown (1912a) have made clear the nature of stepping showing that it is a fundamental activity of the nervous system,—rhythmic in nature and dependent upon two peripheral, antagonistic forces, one tending toward flexion, the other toward extension. Now one force, now the other becomes dominant, the antagonistic action being successively in a state of inhibition. The play of these centers balanced one against the other leads to alternate flexion and extension of the extremities.

Moreover, this tendency to rhythmic activity is, as shown by the experiments of Sherrington, a property of the isolated spinal cord. He obtained evidence which indicated that the proprioceptive fibers from the muscles carried the afferent impulses responsible for reflex stepping. Philippson (1905) had formerly believed that stimuli from the cutaneous surface of the body, received when the foot-pad was in contact with the ground, elicited the rhythmic beats of the extremities. Sherrington showed that stepping ensued after the skin of the foot-pads or of the entire leg as well had been removed. Graham Brown (1912) afterward demonstrated that even the integrity of the proprioceptive path-

way was not essential. He exposed and quickly transected the thoracic portion of the spinal cord in animals whose hind-legs had been completely de-afferented, and observed that first a marked flexion occurred, followed by several rhythmic beats of the hind-legs which finally came to rest in a position of extreme extension. The rhythmic tendency in this experiment, at least, seemed to be maintained on the motor side of the reflex arc. Brown considered that under certain conditions such as asphyxia these motor centers might be influenced to rhythmic activity by substances contained in the blood stream after the manner of the respiratory center. In further experiments to test this theory of rhythmic coördination on the motor side without the intervention of the afferent portion of the reflex arc, he showed that rhythmic movements of the extremities occurred in cats at a depth of narcosis at which the afferent pathways were apparently no longer excitable (Graham Brown, 1914).

Although orderly rhythmic stepping occurs in the spinal animal this does not exclude a normal control of the activity by centers in the brain. Thiele (1905) found that after transection of the cerebral axis just behind the thalamus spontaneous progression no longer occurred. Mella (1923) believed that in cats there was in the subthalamic region an area, the removal of which caused cessation of automatic locomotor movements associating fore- and hind-legs. Laughton (1924) observed that in the adult cat and dog two-thirds of the thalamus must be intact in order that spontaneous progressive movements might occur. The evolution of motor centers in the fore-brain and particularly in the cerebral cortex undoubtedly plays an increasing part in the regulation of the rhythmic beat in higher mammals. The possibility of a center in the diencephalon controlling progressive movements will be considered in another place.

General characteristics of decerebrate rigidity. Sherrington in 1896–97 performed a fundamental experiment in the study of the central control of the postural reflex when he observed that if transection of the neural axis was made at the level of the crura cerebri in experimental animals, there ensued an outspoken tonic rigidity in certain groups of skeletal muscles, especially in those of the dorsal aspect of the neck and tail and the extensor surfaces of the limbs. He compared these preparations with animals (monkeys) in which the cerebral axis had been transected in the lower half of the bulb (1897–98). In the latter case the monkey hung from the suspension points with deeply drooped neck and tail, its pendent limbs flaccid and slightly flexed. On

giving the hand or foot a push forward and then releasing it, the limb swung back into and somewhat beyond the position of its equilibrium under gravity and oscillated a few times backward and forward before finally resuming its original position.

On the other hand, after typical decerebration (removal of cerebral hemispheres, basal ganglion and the anterior portion of the mid-brain) the animal hung with the fore-leg thrust backward with retraction at shoulder-joint, straightened elbow and some flexion at the wrist. The hand of the monkey was turned with its palmar surface somewhat inward. The hind-legs were similarly kept straightened and thrust backward; the hip was extended, the knee very stiffly extended and the ankle somewhat extended. The tail was held straight and horizontal or often stiffly curved upward. There was a little opisthotonus of the lumbo-sacral region. The head was lifted against gravity and the chin tilted upward under the retraction and backward rotation of the skull. When the limbs or tail were pushed from the pose they had assumed, considerable resistance to the movement was felt and on being released they sprang back at once to their former position and remained there for a time more stiffly than before. In the monkey, cat, dog, rabbit and guinea pig the effect upon the fore-leg seemed more intense than on the hind. The knee was the principal joint affected in the hind-leg. The wrist and ankle were comparatively slightly implicated, the ankle more than the wrist. Sherrington was never able to satisfy himself that the digits were involved at all.

Sherrington further found that when this extensor hyper-tonus was absent or very slight it could soon be developed by passive movements of the part. For example, passive flexion and extension of the elbow sufficed to bring out in a few seconds a high degree of tonus of the extensor muscles which, after continuing a short time, tended to relax again. There was no obvious tremor in the spasm in the earlier hours of its continuance, later it sometimes became tremulant. Chloroform and ether, if carried far, quite abolished the rigidity which, on interrupting the administration, rapidly returned.

Sherrington called this condition following removal of the fore-brain "decerebrate rigidity." The attitude of the animal was subsequently shown to be essentially the reflex posture of standing; after cutting off inhibitory influences from the fore-brain, the anti-gravity reflex became accentuated. As regard the level of the section,—"decerebrate rigidity is a condition which ensues on removal of the fore-brain by transection at any of the various levels in the mesencephalon or in the thalamen-

cephalon in its hinder part" (Sherrington, 1906). Later he stated that "by a decerebrate preparation is understood one in which the whole brain in front of the posterior colliculi has been removed" (Sherrington, 1910).

The technique used in performing the operation has been varied from time to time by different investigators. Sherrington transected the brain-stem with a knife or spatula after the carotids were tied and the calvarium opened on one side. The vertebral arteries were compressed digitally until the bleeding was controlled. Later Miller and Sherrington (1916) perfected the guillotine method by means of which numbers of specimens could quickly be prepared for use in the physiological laboratory. Pollack and Davis (1923) located the basilar artery by exposing it in the roof of the mouth and ligated it at different levels, also tying the carotids, thus making the anterior portion of the brain anemic. By injecting a solution of methylene blue they were able to demarcate the level of the lesion since the anemic portion of the brain stained an intense blue.

PERIPHERAL MECHANISM OF POSTURE. The extensor tonus characteristic of the postural reflex (decerebrate rigidity) is controlled very little by centers in the spinal cord (Sherrington, 1910 and 1915). A cat with the spinal cord transected in the lower thoracic region weeks or months previously, if placed symmetrically in the standing posture with the hind-legs together and the fore-legs supported by an assistant, is capable of maintaining the posture and supporting the weight of the hind-quarters even for minutes at a time. Spinal standing, however, is subject to sudden lapses and there is little latitude in the exact pose of the limbs compatible with standing. In short there is in the tonic reflex which maintains standing in the purely spinal condition little of the plasticity which the normal cat exhibits. The reflex tonus which executes the posture of standing in the decerebrate preparation has "plasticity," i.e., capacity for local modification in regard to the length of this or that particular muscle or muscle groups without disturbance of the tonic tension of the muscle.

The source of the afferent impulses controlling the postural reflex has also been elucidated by Sherrington (1897–98). He found that, when the rigidity was maintained at its extreme height, careful section of the afferent roots, previously laid bare and prepared, caused the limb to fall at once into flaccidity. From the stiffly extended position it dropped to the slightly flexed position assumed when flaccid under gravity. Moreover, the rigidity developed very imperfectly or not at all after the

afferent fibers had been severed a number of days prior to the transection of the brain stem.

Afferent nerves from muscles exercise a great local influence on the decerebrate rigidity. Electrical stimulation of the central end of a nerve trunk distributed purely to the muscles (for example, the hamstring nerve of the cat) produced immediate relaxation of the rigid extensors of the knee. On discontinuing the excitation the extensor rigidity of the knee returned. A ligature drawn tightly around this nerve kept the knee on the ipsilateral side relaxed by acting as a continual slight stimulus. The extensor rigidity of the knee on the crossed side seemed, on the other hand, somewhat increased. Mere section of the hamstring nerve did not prevent the development of rigidity of the knee. Impulses from the muscles both excite and inhibit the extensor hyper-tonus. The afferent impulses from practically the whole skin of the limb may be severed without obviously affecting the reflex.

The source of the centrifugal influences maintaining the postural reflex has been traced to the muscles which themselves exhibit the tonic contraction. The source of the reflex is in itself therefore of a unitary functional character. Local modifications of the plasticity are given by this intrinsic proprioceptive reflex. To demonstrate more clearly this local modification Liddell and Sherrington (1924) investigated the myototic or muscle-stretch reflexes. They showed that the slightest pull on a knee extensor muscle of the decerebrate cat was sufficient to produce a contraction in it. This was reflex and purely proprioceptive, its receptors lying in the fleshy portion of the muscle. The reflex contraction had a short latency and was limited to the portion of the muscle which had been stimulated.

Walshe (1924) demonstrated the importance of afferent proprioceptive influences in the maintenance of certain pathological types of rigidity seen in the clinic by injecting 8 to 25 cc. of sterile 1 per cent procain into the biceps, triceps and fore-arm muscles and studying the effect of de-afferentation of these muscles in cases of paralysis agitans. This abolished the rigidity but left the voluntary power unimpaired.

Afferent impulses from other sources undoubtedly influence the postural reflex. Ewald (1892) believed that destruction of the otic labyrinth reduced the tonus of the skeletal musculature of the ipsilateral half of the body. Sherrington (1897–98) planned an experiment to test whether section of the N. Octavus would effect the development of decerebrate rigidity. In the monkey the eighth cranial nerve on the left side was accordingly cut intra-cranially between its surface origin

and the internal auditory meatus. Five hours later the cerebral hemispheres were removed under anesthesia. Decerebrate rigidity then quickly set in and developed with about equal rapidity on the two sides. Decerebrate rigidity appeared as usual when one labyrinth was removed eight days and the other three days before decerebration. The N. Octavus can, it is true, regulate, adjust and modify the reflex standing of the decerebrate preparation. Weed (1914) further found that removal of both internal ears had no effect upon the rigidity after it had developed. Further support of the view that the control of the labyrinth was not essential for the rigidity was given by the fact that section of the inferior cerebellar peduncles had no effect on the rigidity.

The tonic postural reflex still goes on when the decerebrate cat is placed on its side (Sherrington, 1910). That the animal still stands when laid on its side seems an extravagant statement. It merely implies that in the preparation, the standing posture is largely beyond the control of the otic labyrinths.

Magnus (1912) demonstrated that flexion, extension or rotation of the neck of a decerebrate animal produced changes in the pattern of the plastic tone. For example strongly depressing the head decreased the tone in the fore-legs and increased the tone in the hind-legs, as if the cat were looking under a shelf, while elevating the head (strong dorso-flexion of the neck) increased the tone in the fore-legs and decreased the tone in the hind-legs as if the cat were looking up on a shelf. Rotating the head toward one side increased the tone in the extremities of that side and decreased it in the contralateral. The sources of the impulses producing these changes in tone pattern Magnus believed were the otic labyrinths and particularly proprioceptive impulses from the neck muscles.

The sum total of postural reflexes in the decerebrate animal Magnus speaks of as the standing reflex while additional reflexes of the midbrain preparation he calls position or righting reflexes (Magnus, 1924). These latter are analyzed into five groups according to their source and the muscles upon which they act: 1. Optic (probably retinal righting reflexes). 2. Labyrinthine righting reflexes. 3. Righting reflexes arising in the deep tissues of the body wall and acting on the head. 4. Righting reflexes arising in deep structures of the neck and acting on trunk and limb muscles. 5. Righting reflexes arising in deep muscles of the body wall and acting upon limb and trunk muscles.

Magnus (1925) believes that the nervous influences affecting posture can be otherwise grouped under four headings, labyrinthine, proprio-

ceptive, exteroceptive (chiefly pressure) and influences from the teleoceptors (eye, ear and nose). Motor stimuli from the cortex cerebri tend to inhibit decerebrate rigidity but are much less important than from the red nuclei. In man attitudinal reflexes (labyrinthine and neck) cannot easily be demonstrated. On the other hand it is only in higher mammals that it is possible to show the presence of optic righting reflexes.

The centers for the eye reactions in the brain stem are found between the entrance of the sensory nerves concerned and the eye-muscle nuclei; those for the other righting reflexes extend as far down as the pons and upper part of the bulb. Rademaker (1924) has shown that the red nucleus is the center for labyrinthine righting reflexes and for body righting reflexes acting on the body but not for body righting reflexes acting on the head. The efferent path of the two first named reflexes is the rubro-spinal tract; the afferent tracts have still to be found. All these postural reflexes Magnus found present and undisturbed after total extirpation of the cerebellum.

Spiegel and MacPherson (1925) found that the afferent fibers controlling the neck righting reflexes of Magnus run in the posterior roots of the upper three cervical nerves and end in the first two cervical segments of the cord. The descending pathways do not lie in the anterior or posterior columns of the cord but run from the uppermost cervical segments in the lateral columns of the same side. They are in close relationship with other tonus regulating systems in this column but seem to be located predominantly in the most dorsal portion of the lateral column. Beritoff (1915) investigated the effect of compounding the ordinary "nociceptive" limb reflexes against the labyrinthine and cervical tonic reflexes. The investigations of Muskens (1914) seem to indicate clearly that fibers which lie in the posterior longitudinal fasciculus have an important part in the control of the righting reflexes of Magnus.

Magnus' distinction between standing and righting reflexes gives a new and interesting viewpoint useful in his experimental approach to this problem. The righting reflexes apparently bear the same relation as progressive movements to the standing or the postural reflex as we have chosen to call it. Thus progressive or righting reflexes should be considered under the heading of the normal control of posture regulating the extensor tone in such a way that the animal is able to strike and maintain useful postures and attitudes. Correlating, then, Magnus' classification with the present discussion, we will discuss the reflex path-

ways producing postural tone and also the influences that control or inhibit it.

SPINAL PATHWAYS OF POSTURAL REFLEX. The nuclei and pathways of the postural reflex in the brain are not at present well understood and the evidence conflicts at many points. The experimental information becomes more intelligible when it is realized that practically all portions of the central nervous system have a part in this tonic control. The current information concerning the central control of the postural reflex has been obtained from stimulation and ablation experiments testing the effect both of increasing and decreasing extensor tonus.

Sherrington (1897–98) showed that the efferent pathway of the reflex could not be the pyramidal tract since section of one lateral half of the bulb in the lower half of the floor of the fourth ventricle and just above the level of the decussation of the pyramids, abolished rigidity on the same side as the section. And further transverse severance of the lateral portion only of this part of the bulb without interference to either pyramidal tract produced similar abolition of the rigidity of the ipsilateral legs.

The pathway of afferent impulses in the spinal cord is not clearly understood. Sherrington (1897–98) found that section of the dorsal columns of the spinal cord did not abolish the rigidity. Section of one lateral column in the upper lumbar region abolished the extensor hypertonus in the ipsilateral hind-limb. Likewise, section of one ventrolateral column in the cervical region destroyed the rigidity in the fore and hind-legs of the same side. Such a cut might easily sever the efferent as well as the afferent system of fibers.

Thiele (1905) was able to cut the direct cerebellar tract (Flechsig) as well as the dorsal columns without any influence upon the extensor tone. He assumed that the afferent proprioceptive influence was merely local and that afferent ascending fibers were not required for the maintenance of the reflex. This opinion is hardly consistent, however, with current neurological thought.

Weed (1914) likewise eliminated the direct spino-cerebellar fibers (Flechsig) as the afferent pathway for he found that cutting the inferior cerebellar peduncles (restiform bodies) did not diminish the existent extensor hyper-tonus. After severing the superior cerebellar peduncles (brachia conjunctiva) decerebrate rigidity entirely disappeared in a majority of his animals. He therefore believed that the afferent impulses ascended to the brain in the ventro-lateral cerebellar tract of Gower. Not all of these fibers end in the cerebellum; some terminate

in the corpora quadrigemina and others travel forward to the thalamus (Mott). In the two cats in his series in which extensor hyper-tonus was not entirely lost after cerebellar ablation he thought it possible that the fibers ending in the mid-brain were sufficient to carry the afferent impulses. Bazett and Penfield (1922) found that in all their chronic preparations showing extensor hyper-tonus Gower's tract was uninjured.

Spiegel (1924) found that section of the antero-lateral columns of the spinal cord gave rise to hypo-tonicity of the ipsilateral legs of the cat without interfering with ordinary voluntary movement. This decrease of tone he attributed to the loss of afferent proprioceptive and of extra-pyramidal influences. The operation appeared to counteract the hyper-tonus of decerebrate rigidity.

From the previous discussion it is clear that the pathway of afferent nerve impulses producing and controlling the postural reflex has not been demonstrated in the central nervous system. The evidence now available suggests that fibers in the tract of Gower ending in the cerebellum, mid-brain and thalamus carry sensory impulses that augment the extensor tonus.

THE CEREBELLUM AND THE POSTURAL REFLEX. Sherrington (1897–98) pointed out that mutilations other than ablation of the cerebral hemispheres induced phenomena of extensor rigidity bearing superficially at least much resemblance. After median section or removal of the cerebellum a rigidity often but not always sets in (see Luciani, 1915). That these two conditions were identical he was not convinced. The uncrossed nature of the decerebrate rigidity suggested to him a causal connection between the cerebellum and the rigidity possibly through Deiter's nuclei which receive large efferent connections from the cerebellum. On the other hand the crossed cerebello-cerebral and cerebro-cerebellar paths, Sherrington thought, might form a circuit whose function would be upset in much the same way whether cerebellar or cerebral ablation be performed. Sherrington found that decerebrate rigidity sometimes persisted after removal of the cerebellum if the latter ablation be performed without any serious hemorrhage. Those allied forms of extensor rigidity further resembled decerebrate rigidity in similarly being broken down by appropriate central and peripheral excitations, among the former of which are to be included excitations applied to the Rolandic area of the cortex cerebri.

Thiele (1905) tested the effect of interruption of the possible reflex connections between the cerebrum and cerebellum having a part in the control of the postural reflex. The afferent channel for this circuit

might be the afferent tracts of the cord acting through the cerebellar cortex, the dentate nucleus, the superior cerebellar peduncle (brachium conjunctivum) to the crossed rubro-thalamic nuclei and cerebral cortex. To cut this pathway Thiele drew forward one cerebral hemisphere and cut the superior peduncles of the cerebellum after chipping away a part of the bony tentorium. The result of this section was a slight increase in the rigidity already present after manipulation of the tentorium. The rigidity was always increased upon subsequent transection of the mesencephalon. It therefore appeared that as far as decerebrate rigidity was concerned the cerebellum does not exert a marked controlling influence over the opposite thalamus. The possible efferent channel would be the cortico- and thalamo-ponto-cerebellar fibers. In two cases fairly well marked bilateral rigidity with more marked homolateral effect occurred as a result of superficial incision into the side of the left crus whereby some fibers of the cerebro-pontine tract were divided.

Unilateral separation of Deiter's nucleus from the medulla or division of the vestibulo-spinal tract as it passes down through the medulla produced relaxation of the extensor rigidity on the ipsilateral side according to Thiele (1905). In all cases the relaxation was not complete and a certain amount of rigidity sometimes occurred after an interval. Thiele considered that the source of the rigidity was Deiter's nucleus. Piecemeal removal of the cerebellum by horizontal slices produced no influence on the pre-existing extensor rigidity until Deiter's nuclei were included in the section when, as before, the rigidity was abolished.

Thiele also believed that the cerebellar cortical cells exert an inhibitory action over Deiter's nuclei of both sides but with more marked effect ipsilaterally. Simple removal of the tentorium produced extensor rigidity of the same nature as after mesencephalic transection. Removal of the tentorium over one lobe of the cerebellum produced bilateral rigidity which was usually more marked on the same side. If the tentorium was not previously cut away, then removal of one lateral lobe of the cerebellum by successive sagittal slices caused at first development of a slight degree of bilaterial rigidity which was usually at first more marked on the contralateral side but subsequently increased ipsilaterally. In other cases the ipsilateral rigidity was the most marked from the beginning. Rigidity could also be produced by division of the superior vermis.

The rôle of the cerebellum in the control of the postural reflex is very obscure. Both its excitatory and inhibitory function in the regulation

of postural tone must be considered. Löwenthal and Horsley (1897) and Sherrington (1897–98) demonstrated that stimulation of the superior vermis produced a marked inhibition of extensor tone. Ferrier and Turner (1893) and Russel (1894) had shown that fibers from the vermis degenerate toward the vestibular and Deiter's nucleus. They also believed that the superior vermis exerted a control over the red nucleus by way of the roof nuclei and superior peduncle.

Sherrington (1897–98) found that afferent proprioceptive impulses exerted both pressor and depressor effects upon the postural reflex. In this connection Ingvar (1923) observed that the spino-cerebellar fibers end almost exclusively in the anterior and posterior lobes of the cerebellum while the middle lobe receives none. These areas corresponded exactly with two regions where Bremer (1922) located centers from which extension of the extremities could be inhibited by the application of mild electric currents. Bremer found that these excitable zones had very distinct boundaries. The area in the anterior vermis did not exceed the middle of the central lobe. The posterior border was about 1 to 2 mm. from the primary fissure. The other inhibitory center was situated at the level of the pyramis. Spino-cerebellar fibers originate, according to Ingvar, from all segments of the spinal cord. They are not segmentally represented in the cerebellar cortex suggesting that these regions have a function correlated to all spinal segments, that is, to the body or organism as a whole. The lingula of the anterior lobe and the uvula, nodulus and flocculus of the posterior lobe receive direct fibers from the vestibular ganglion.

Weed (1914) devised a number of experiments to ascertain whether the cerebellum was an essential link in the reflex pathway producing postural tone. Exposure of the posterior part of the cerebellum just before the brain-stem was transected interfered markedly with the subsequent development of extensor rigidity. On the other hand, exposure of the posterior cerebellar cortex after strong rigidity had appeared produced a marked accentuation of the hyper-tonus which lasted but a few minutes, the cat then resuming the degree of rigidity present before the second operation. A hot saline swab (45°C.) applied to the posterior cerebellar cortex occasioned a short but very marked increase in the existent rigidity. Conversely the application of a cool saline swab (16°C.) diminished the rigidity.

Portions of the cerebellum were removed after strong decerebrate rigidity had developed (Weed, 1914). The exposure and ablation of one lateral lobe of the cerebellum, leaving the vermis region covered by

dura and bone and avoiding injury to the vestibular nuclei caused a diminution of the rigidity in the ipsilateral fore- and hind-leg. The rigidity of the trunk and tail was unaffected. Removal of one half of the cerebellum caused a diminution in the rigidity of the tail as well. The ablation of the whole of the vermis, exposing the ventricular floor beneath did not effect the development of a bilateral extensor rigidity of the customary degree.

In a considerable number of cats the whole cerebellum was removed at an interval after the initial procedure of decerebration and the development of a constant degree of rigidity. Care was taken to avoid injury either to the peripheral vestibular pathway or its central nuclei. This was easily done by elevation of the whole cerebellum away from the ventricular floor with subsequent section of the peduncles laterally.

The results were in agreement except in one instance. Section of the cerebellar peduncles was always followed by a marked accentuation of the existent rigidity. Immediately after this accentuation the extensor tonus diminished rapidly. The time required for disappearance of all traces of extensor rigidity varied considerably but ordinarily it was gone within twenty minutes after cerebellar extirpation. In one exceptional case the rigidity could be demonstrated for over an hour. Miller and Banting (1922) also found complete loss of extensor hypertonus after removal of the cerebellum.

In one cat prepared by Weed, the cerebellum was completely removed four weeks before transection of the brain-stem. During the first few days following the cerebellar operation the cat showed marked extensor rigidity in the fore- and hind-legs equal on both sides. This disappeared and did not reappear. Decerebration was followed within two or three minutes by the development of extensor rigidity in the elbows and wrists equal on both sides. This rigidity increased and in the next ten minutes attained the normal degree for such a period after decerebration. There was later considerable diminution in the hyper-tonus so that little remained a half-hour after decerebration and at the end of three hours and a half the animal was quite flaccid although breathing spontaneously.

Weed (1914) then thought it important to determine by which peduncle the impulses producing the hyper-tonus were carried to and from the cerebellum. Section of the inferior (corpus restiforme) and middle (brachium pontis) peduncles bilaterally had no appreciable effect upon the existent extensor rigidity. Severance of both superior cerebellar peduncles (brachia conjunctiva) resulted in a marked initial accentuation followed by a total loss of all rigidity. Section of one

superior peduncle resulted in an initial augmentation of the rigidity on the same side followed by a marked diminution of the ipsilateral rigidity but never a total loss. The rigidity of the contralateral extremities was apparently not affected.

Miller (1926) suggests that the cerebellum is constituted of a group of reflex centers which, in response to streams of proprioceptive impulses from the labyrinths and the body generally, discharge motor influences which both produce and regulate the postural tone of the muscles of the body. Recognizing that the cerebellum is a tonus mechanism influencing tonus in the way of augmentation or inhibition it must be thought of as being concerned with postural tone since that is the only kind which exists in the body. It regulates and refines the crude adjustments of tonus affected by spinal and prespinal centers.

Weed's evidence thus led him to the conclusion that the cerebellum forms an important if not essential link in the control of the postural reflex. The work of Miller and Banting (1922), Camis (1923), Miller (1926) and others tends to support the hypothesis. On the other hand Magnus and Rademaker (1924) think that the cerebellum has no part in the production of extensor tone and that decerebrate rigidity develops with great intensity after total ablation of the cerebellum. Weed suggests that the time element is an important factor in these experiments that has not been given proper consideration. If the cerebellum is removed coincident with decerebration little or no extensor hyper-tonus can be demonstrated. But when a period of days or weeks elapses between the ablation of the cerebellum and decerebration, rigidity of great intensity may appear. It is possible that after removal of the cerebellum other structures in the brain stem may partially assume its function. However this may be, there are few data available at the present time concerning the part played by the cerebellum in the production or control of postural tone. In such experiments the technique of the investigator plays an enormous rôle.

MESENCEPHALIC CENTERS INVOLVED IN POSTURAL REFLEXES. To outline further the efferent pathway the mid-brain was sectioned in the mid-line from the region of the anterior colliculi caudad to the anterior limit of the pons after strong rigidity had developed (Weed. 1914). The cut was followed by an immediate initial accentuation of the rigidity and a later marked diminution so that in the course of a few moments the degree of rigidity remaining was very small. This indicated that there was a partial rather than a complete decussation in the tegmentum of the mesencephalon. The cut severed not only the efferent paths

from the cerebellum but also any decussating fibers from the mid-brain to the cord.

Removal of one-half of the mid-brain to the cephalic border of the inferior colliculus caused a considerable diminution of the rigidity on the opposite side with practically no effect upon the same side (Weed, 1914). Later unilateral removal to the caudal end of the inferior colliculus diminished that rigidity to an enduring very slight degree. Total transection of the mid-brain to the caudal border of the inferior colliculi coinciding with the cephalic limit of the pontine nuclei produced a short accentuation of rigidity followed very quickly by a total loss of all rigidity with the assumption of a flaccid flexor attitude.

Weed (1914) therefore believed that the caudal portion of the mid-brain must be intact if extensor rigidity were present. Moreover, the reflex path crossed at least once in the mid-brain. He suggested that the afferent fibers travel in the ventro-lateral tract of Gower and many of them reach the cerebellum by way of the superior peduncles Efferent tracts from the cerebellum also travel in the superior peduncles. Since the red nucleus is the largest group of motor cells in the mid-brain region, Weed hypothesized that these cells give rise to the efferent pathway to the cord. The fibers from the cerebellum cross in the mid-brain to the opposite red nucleus and the rubro-spinal tract also decussates, in large part at least, early in its course. Because of the prominent part that the red nuclei play in a discussion of the postural reflex their histological structure and connections may well be briefly reviewed.

Microscopically, the red nuclei of lower vertebrates are composed of large cells of motor type from which arise the rubro-spinal tracts. Higher in the phylogenetic scale small cells appear in the red nucleus and it is possible to distinguish orally placed groups of smaller cells, the nucleus parvi-cellularis or chief nucleus from the more caudal giant cells or nucleus magno-cellularis. The small celled nucleus develops along with the thalamus and the cerebral cortex and is connected with them. In anthropoids and man this nucleus of small cells makes up the greater part of the red nucleus while the giant cell part has become very small and as a result the rubro-spinal tract has greatly diminished in volume. Because of the great increase in number of the small cells, the red nucleus of man is almost twice as large as that of the cat in proportion to the vertical diameter of the mid-brain in each species. In the cat the red nucleus is circumscribed by a feltwork of fibers. The caudal two-thirds is made up of giant cells, the cephalic one-third of

small cells according to Hatschek (1907). The phylogenetically younger nucleus parvi-cellularis is in relation with the nucleus dentatus of the cerebellum while the older nucleus magno-cellularis is interposed on the pathway of the cerebellar nuclei globosus and emboliformis above and the descending rubro-spinal tract. This close anatomical relationship of nucleus magnocellularis with the cerebellum and Monakow's tract constitutes a primitive path of nerve impulses for automatic movements. Other tracts converge on the red nucleus. Fibers arise in the frontal cortex and pass by way of the red nuclei to the tegmentum of the mid-brain and formatio reticularis of the pons from which they are supposed to send relays to the spinal cord. Wilson has shown that there is a considerable tract of fibers from the globus pallidus which passes across Forel's field to the anterior capsule of the red nucleus.

The principal red nucleus efferents are the rubro-spinal tracts of Monakow which cross the mid-brain in the fountain decussation of Forel. This tract arises wholly in the giant cells according to Cajal. Von Monakow describes some fibers as coming from the cells of medium-size. Certainly the tract arises chiefly from the nucleus magno-cellularis and comparative anatomical studies show that this part of the red nucleus varies directly in size with that of the tract in most animals and both are small in man. Efferent fibers of importance also pass from the red nucleus to the tegmentum and formatio reticularis as well as to the thalamus and frontal cortex (Bazett and Penfield, 1922).

There is an increasing amount of evidence that a certain modified type of extensor hyper-tonus is often present after section of the brain-stem below the red nuclei. Cobb, Bailey and Holtz (1917) suspended decerebrate cats so that levers attached to the extensor muscles would record the tonic changes. Decerebration was performed at different levels and the effect upon the extensor rigidity studied; the level of the section was confirmed histologically. In the animals with red nucleus intact the rigidity was strong and constant. In the second group of cats where the red nuclei were absent there was a kind of extensor hyper-tonus but it was more variable and depended on afferent stimuli being frequently applied to the limbs. This rigidity only appeared when induced by massaging the muscles or by moving the limbs passively. The extension lasted but two to ten minutes and then must be renewed by some similar procedure. When the transection was further back in the region of the trapezium and vestibular nuclei all tonic extension was lost.

Bazett and Penfield (1922) prepared decerebrate cats with sterile

technique and kept them alive for days by placing them in a warm box or water bath at a constant temperature. For a time after operation the extensor rigidity was constant but after four or five days flaccid flexion of the legs was often present and the extensor rigidity could only be demonstrated after passive manipulation of the extremities. Kicking and running reflexes were common in the chronic animals after the first few days. Rigid semi-flexion of the fore-legs was also commonly seen. Careful histological study of the post-mortem material led the authors to state that the red nucleus had no part in the control of the postural reflexes since, in many of their preparations, the red nuclei were partially or totally destroyed. But post-operative hemorrhage and infection, just as it evidently produced changes in the reflex behavior of the animals, may have caused a secondary injury to the mid-brain region. The later behavior of the cats was certainly not typical of decerebrate rigidity; they were extremely active animals and extensor hyper-tonus could only be demonstrated after passive movements of the extremities. These animals might better be considered as typical of bulbar rather than of decerebrate preparations. Bazett and Penfield's results appear to verify, in general, the findings of Cobb, Bailey and Holtz (1917) that the extensor hyper-tonus seen after removal of the red nucleus is different from that seen when the red nucleus is intact. This fact however, Bazett and Penfield were not willing to affirm.

Pollack and Davis (1924) using the anemic method described by them, decerebrated cats just cephalad to the exit of the fifth nerve. In these animals decerebrate rigidity appeared. The cats were not immobile, however, and many pseudo-affective reactions could be easily demonstrated.

Magnus and Rademaker (1924) reported that an animal with an intact mid-brain differed from a decerebrate animal by the absence of decerebrate rigidity and the presence of normal postural reflexes. After transection in the rabbit through the center of the right superior corpora quadrigemina and the posterior third of the left and through the cerebral peduncle just posterior to the mammillary body below, the postural reflexes were normal and no decerebrate rigidity resulted. Sections anterior to the red nucleus produced no rigidity. After all sections which injured the red nucleus decerebrate rigidity was demonstrated and labyrinthine postural reflexes were abolished. The opinion of Magnus and Rademaker differs radically then from that of Weed since the former believe that the red nucleus normally exerts an inhibitory control over the postural reflex without any excitatory com-

ponent and that it is only after this control is removed that decerebrate rigidity results.

Rademaker (1924) in his recent book states that the "thalamus animal" preserves normal tonus in its muscles and can coördinate its movements; it is consequently able to stand, sit, walk and jump and if it falls it can regain its normal position immediately. The decerebrate animal has lost all its righting reflexes except the neck-righting reflex which is situated farther caudad than other postural reflexes. Tonus is unaltered and the righting reflexes are intact after transection through a plane extending from the cephalic border of the anterior corpora quadrigemina to the pes pedunculi, 2 mm. oral to the emergence of the oculo-motor nerve and just cephalad to or through the oral ends of the red nuclei. Rigidity develops after transection-through a plane from the posterior part of the anterior colliculus to the peduncles caudad to the emergence of the third nerve. This section passes through the caudal end of the red nuclei.

Rademaker destroyed the red nuclei by stab wounds in intact animals as well as after removal of the fore-brain and in thalamus animals. When rigidity developed and the righting reflexes disappeared Forel's decussation or the red nuclei were always injured. In cats, rigidity was less extreme when the fore-brain and cortico-spinal tracts were intact although in rabbits it did not matter whether the fore-brain was removed or not. A striking effect was seen in the rabbit when a fine thread was inserted through the middle of the mid-brain behind Forel's decussation. The animal could still walk and jump normally but when the thread was drawn oralwards so that it injured Forel's decussation, the animal at once became rigid, fell and lost its labyrinthine and body-righting reflexes. The red nuclei and rubro-spinal tracts are part of the mechanism for the maintenance of normal tonus, for labyrinthine righting reflexes and body righting reflexes and the red nuclei can still maintain these functions after removal of the fore-brain, corpora striata, optic thalamus and most of the oral part of the mesencephalon and corpora quadrigemina. The red nucleus is able to regulate the labyrinthine righting reflexes after ablation of the cerebellum. Magnus and Rademaker then would exclude the cerebellum and red nucleus from any part in the production of the postural reflex but they ascribe to the red nucleus an inhibitory control of this extensor tonus.

A majority of Magnus and Rademaker's experiments were performed upon rabbits. There is some evidence that in these animals the control of posture by centers above the mid-brain is not as highly developed as

in other laboratory animals, the cat and dog for instance. This is suggested in Rademaker's work, since he found that in cats the rigidity following severing of the rubro-spinal fibers was increased by subsequent removal of the fore-brain. Laughton's (1924) studies also give this same impression. Laughton disagrees with Rademaker and believes that decerebrate rigidity is due to the removal of a cerebral influence rather than to removal of a structure in the region of the red nucleus. Certainly, as will be demonstrated more clearly further on in this discussion, most investigators are in accord that extensor hyper-tonus may be demonstrated after transection of the brain stem through or even above the diencephalon.

Mussen (1927) produced local lesions of the red nucleus with the "steriotaxic" instrument or Horsley-Clarke apparatus. After destruction of the posterior pole of the red nucleus on one side, no rigidity developed nor was there any disturbance of righting reflexes. Marchi sections showed a complete degeneration of the rubro-spinal tract to the sacral region of the spinal cord. Moreover, a slight unsteadiness of gait which lasted two days was the only abnormality observed after bilateral injury to the nucleus magno-cellularis. During the remaining eighteen days of life the gait was normal and the reflexes, sense of position and muscle tone were not affected.

The nucleus parvi-cellularis on one side was destroyed by Mussen without injuring the nucleus magno-cellularis. In sections stained by the Marchi method there could be located a well-marked tract of degeneration which crossed immediately under the nuclei and could be followed into the cervical portion of the cord. In this animal the righting-reflexes were temporarily lost; the animal could be placed in any position—right side, left side or on its back and it would remain in this position. After the fifth day the abnormality gradually improved; the disturbance in the righting reflex disappeared and through the second and third weeks the only symptom that remained was some unsteadiness in balancing. At times the head was turned slightly to the right.

Graham Brown (1913) stimulated the cut surface of the mid-brain of monkeys following high decerebration (slightly anterior to the superior colliculi). After transection of the brain at this level the animals were not entirely immobile; slow rhythmic flexion and extension of the arms occurred at intervals. The hind-legs demonstrated the immobility of extensor rigidity more than the fore-legs. Stimulation of an area including the nucleus ruber and the posterior longitudinal fasciculus on one side of the mid-brain was accompanied by the assumption of a

definite posture on the part of the animal. The back of the head was twisted toward the same side and the face away from it: the arm of the same side was flexed; that of the opposite side extended. The hind-leg of the same side was extended and the contralateral flexed. The tail was bent to the same side. When stimulation had ceased this posture was maintained for a considerable period.

Two bipolar electrodes, attached to different circuits, were used by Graham Brown so that, at will, two points could be stimulated simultaneously. Bilateral stimulations were synchronously compounded in such a manner that the ipsilateral effect interrupted a contralateral "background" or the contralateral an ipsilateral "background." When compounded against an ipsilateral "background" (flexion), the effect of stimulation of the contralateral side (extension) was to produce relaxation of the "background" flexor contraction. When compounded against a contralateral "background" (extension) an interrupting ipsilateral stimulus (flexion) evoked extensor relaxation and flexor contraction. The ultimate effect depended upon the relative strength of the opposing stimuli. These reactions occurred even many months after division of the dorsal spinal roots of the fore-leg. Brown (1915a) later obtained similar results on stimulation of the region of the red nucleus in an anthropoid ape. In a discussion of these experiments, the part played by stimulation of the posterior longitudinal fasciculus must certainly receive most careful consideration (see Muskens, 1914).

Weed (1914) found that stimulation of the mesencephalic tegmentum on either of the raphe in the region of the cranial end of the red nuclei with weak current resulted in a contralateral accentuation of the extensor rigidity at the elbow. With a stronger current the contralateral extensor increase was more marked but added to it was a strong ipsilateral flexor rigidity at the elbow.

There is considerable evidence at the present time that Deiter's nuclei are the chief reflex centers concerned in decerebrate rigidity; the experimental proof of this hypothesis is not yet conclusive. Bernis and Spiegel (1925) thought that in addition to the vestibular nuclei, the large cells in the substantia reticularis among which fibers of the antero-lateral columns are seen to end must be considered as playing an important rôle as centers for tonic innervation. A. and N. Zylberlast (1925) suggested that the motor center for the production of decerebrate rigidity was to be found in the inferior olives since destruction of one olive caused flaccidity of extensor hyper-tonus in some cases bilaterally and in one case on the ipsilateral side. The question arises as to whether

the red nucleus cells have a share in the pressor control of the postural reflex as Weed (1914) and later Cobb, Bailey and Holtz (1917) and Langworthy (1926) were led to postulate. There certainly is some proof that this is the case. Electrical stimulation of the cells of the red nucleus produces marked extension of the contra-lateral fore-limb. Moreover, the red nucleus is connected with the great afferent pathway of the cerebellum and all authorities agree that the cerebellum regulates the tonus of somatic musculature. Bernis and Spiegel (1925) believe that in addition to the superior cerebellar peduncle there is a second efferent system (lying in the restiform body and forming the tractus fastigio-bulbaris) which conducts cerebellar tonic impulses to the medulla.

CEREBRAL INFLUENCE UPON POSTURAL REFLEX. It is the belief of many investigators that decerebrate rigidity represents an accentuation of the postural reflex due in part at least to the removal of the inhibitory effect of centers in the fore-brain. Sherrington (1897–98) found that after ablation of one cerebral hemisphere ipsilateral extensor rigidity often developed, presenting thus an opportunity for examining the effect of excitation of the remaining cerebral cortex upon the tonicity of the extensor muscles of the crossed elbow and knee. He was able to locate in the Rolandic area of the monkey an area which gave, markedly and forthwith, inhibition of the contraction of the extensors of the elbow and another cortical area which similarly when excited inhibited the contraction of the extensors of the knee. The areas of the cortex whence inhibition of the active extensors was elicited were not the same areas whence contraction of the extensors was observed but, on the other hand, coincided with areas whence contraction of the flexors could be obtained.

Thiele (1905) believed that decerebrate rigidity did not appear when the level of the section passed cephalad to the posterior part of the optic thalamus. The rigidity thus obtained was not so marked as that which occurred after lesions farther caudad. The most marked rigidity was demonstrated when the section was made at the level of the posterior corpora quadrigemina. It was noted that so long as the level of the cut did not pass posterior to the pontine nuclei the rigidity remained in great intensity. If, however, the level was through the trapezoid fibers the rigidity was absolutely abolished. He concluded that decerebrate rigidity was not due to the removal of a cortical influence but of a thalamic one.

Two pathways were outlined by Thiele (1905); the influence of these

he thought inhibited the rigidity. The first originated in the optic thalamus and its course was outlined by the following experiment. Thiele was able to produce unilateral rigidity following unilateral mesencephalic transections. Not only was ipsilateral rigidity produced as Sherrington described but if the transection was made forward in the region of the optic thalamus and anterior corpora quadrigemina marked contralateral rigidity was demonstrated; the ipsilateral legs were scarcely at all effected and then rigidity in them occurred later and only on manipulation of the limbs. When the lesion was farther back, behind the superior colliculi, ipsilateral rigidity was obtained with only slight contralateral. Thiele concluded that the inhibitory control of the optic thalamus acted directly on the primary motor neurone; that the tract through which this control was executed decussated high in the mesencephalon and was probably a thalamo-rubro-spinal tract. The second inhibitory pathway of Thiele, as has been previously explained, he believed to arise in the cerebellar cortex and to act directly upon the cells of Deiter's nuclei.

Weed (1914) located in the cut surface of the mid-brain a tract of fibers, which on stimulation caused a marked inhibition of the existent rigidity. The current used was so weak that stimulation of the pyramidal fibers gave no response but when the electrode was applied to the mesial fraction of the cerebral peduncles the rigidity entirely disappeared in the ipsilateral limbs and was diminished in the contralateral. The effect, however, was almost entirely ipsilateral as was proved by stimulation of the area on one side after the introduction in the ventral midline of the mesencephalon of a non-conducting plate. The area from which this inhibition could be obtained was quite small comprising only the mesial one-fifth or one-sixth of the crura. Dorsal in this cut surface were the cephalic poles of the superior colliculi while from these the surface sloped ventrally with a slight anterior deflection. The dorsum sellae lay just anterior to the ventral margin, a factor of interference in the elicitation of the inhibitory response due to frequent oozing from the circular sinus.

It was further found by Weed that after cerebellar ablation, with the extensor rigidity still persisting, stimulation of this mesencephalic area was without influence upon the rigidity. The path then must traverse the cerebellum before exercising its inhibitory effect. The middle cerebellar peduncle on one side was sectioned without decreasing in the least the extensor rigidity. Unipolar stimulation had its full effect in causing the inhibition of ipsilateral rigidity but stimulation of

the contralateral area in the crus produced no response. From this it must be concluded that the path in the mesial faction of the cerebral crus ascends to the cerebellum by way of the contralateral middle cerebellar peduncle.

To delimit further the source of the inhibitory tracts the cerebral hemispheres alone were removed from a cat by Weed. An extensor rigidity of customary degree made its appearance in this animal within two minutes after the extirpation of the hemispheres. As the animal gradually recovered from narcosis, walking movements were observed, rhythmic and existing in definite intervals. During the execution of these movements the extensor rigidity was abolished to reappear as soon as the movements ceased. At the mesial anterior portion of the internal capsule an area was made out about 4 mm. from the mid-line which on mild stimulation gave invariably inhibition of the ipsilateral rigidity. The possibility of the inhibitory pathway having exclusively a thalamic origin was thus completely excluded. In this same animal successive cuts were made down to the caudal portion of the mid-brain. The inhibitory pathway was, after every section, identified in the mesial anterior fraction of the internal capsule and in the mesial portion of the crus cerebri.

The inhibitory pathway, Weed suggested, arose from the cerebral cortex, ran caudad in the anterior mesial portion of the internal capsule, continued downward in the mesial fraction of the crus cerebri and probably ended in the pontine nuclei. The second relay carried it from the pons to the cerebellum by way of the contralateral middle cerebellar peduncle whence it was distributed in part at least to the cortex of the anterior portion of the superior vermis.

There is considerable recent evidence, based for the most part on ablation experiments, that the cerebral cortex participates in the normal control of the postural reflex. Dusser de Barenne (1920) described the behavior of two cats whose entire neopallium had been removed and which were kept alive for considerable periods of time. A report of the post-mortem study of the brains was given by Brouwer (1920). The cats for the first few days after operation held the legs in abnormal positions and raised the feet high in walking. There was a loss of deep sensation which diminished but was to some extent permanent.

Warner and Olmsted (1923) found in acute cat preparations contralateral extensor hyper-tonus in the fore- and hind-legs following ablation of one cerebral cortex anterior to the optic thalamus. If the electrically-responsive motor area alone was removed, paralysis of the

contralateral legs was present with no increase in extensor tone. If the area frontalis was also cut away, definite extensor hyper-tonus in the opposite extremities developed. This rigidity was often masked by voluntary movements, and required appropriate methods for its demonstration. Electrical stimulation of the injured area caused inhibition of the rigidity.

Olmsted and Logan (1925) performed similar operations under sterile conditions and kept the the cats alive for several days. They found that the paralysis ensuing on removal of the electrically responsive motor cortex lasted for four or five days. The postural changes present after removal of the area frontalis were most marked in the contralateral hind-leg and could best be demonstrated by holding the animal suspended or with its back against the observer's body. With the area frontalis removed bilaterally coördination was even more seriously impaired.

King (1927) prepared both acute and chronic cat preparations to study further the defects of posture seen after cerebral ablation. The electrically responsive motor cortex was mapped out by unipolar stimulation and the areas removed localized carefully. The cats were kept alive for three months in order to avoid confusion of the results with transitory irritative phenomena. The area whose removal caused contralateral extensor hyper-tonus corresponded histologically with the "intermediate motor area" of Campbell (1905) or the "area frontalis agranularis" of Brodman (1909).

In King's experiments, sensitivity appeared decreased in the affected contralateral extremities in that a strong stimulus was required to elicit flexion of the hyper-extended legs. Moreover, if the cat was suspended by head and tail, the ipsilateral legs clawed the air wildly in an effort to escape; the contralateral legs remained hyper-extended and quiet. These two findings were explained on the basis of the difficulty of overcoming the hyper-extension of reflex standing.

King (1927) further found that after bilateral removal of the area frontalis more marked abnormalities in posture could be demonstrated. For the first few days after the operation the legs tended to become hyper-extended in abnormal positions. Later, the extremities were handled more perfectly but the cats were never able to jump upon or off from a small elevation. When placed upon their backs or made to stand upon the hind-legs the cats would remain quiet in abnormal postures. If the head were suddenly depressed, balance could not be adjusted quickly and the hind-legs would swing up from the ground.

The extensor tonus present in the extremities was of a plastic character. The hyper-tonus could in general be more clearly demonstrated in the hind-legs than in the fore whereas in decerebrate animals the rigidity is usually more marked in the fore-legs. Bermis and Spiegel (1925) thought that in addition to the frontal lobe, centers in the temporal lobe controlled postural tone.

Wilson and Walshe (1914) reported cases of injury to the intermediate motor area in man in which the prominent symptom was tonic perseveration or the inability to relax a given innervation in any muscle group. In all three cases there was a slight but unmistakable involvement of the cortico-spinal tract but the greater the involvement of the cortico-spinal tract, the less marked the tonic innervation. In each case there was slight spasticity of the affected legs. The symptom was evident only during voluntary innervation of the muscular group concerned.

That inhibition of muscular contraction was obtainable by artificial excitation of the cortex was early noted by Bubnoff and Heidenhain (1881) in the dog and by Exner (1882) in the rabbit. This inhibitory effect was later clearly demonstrated in the case of the extra-ocular muscles by the ingenious experiments of Sherrington (1894 and 1905). Sherrington (1906) pointed out that it was difficult by stimulation of the cortex to elicit extension of the knee or closure of the jaw. This does not mean that these muscles are not represented in the cortex or that the cortex is in touch with the flexors alone and not with the extensors. It means that the usual effect of the cortex upon the latter is one of inhibition. This can be clearly demonstrated by the application of strychnine which changes inhibition into excitation. Sherrington thought that the motor innervation of the cerebral cortex should be thought of as phasic, that of the cerebellum as tonic. Little is known at present concerning the mechanism of cerebral inhibitory control.

The cerebral cortex appears then to inhibit and control the postural reflex but whether the hyper-tonus following cerebral injuries is due to overaction of the primary motor neurone or to the removal of an inhibitory control acting through the cerebellum, must first be completely demonstrated before further studies of the pathway are considered. Unpublished experiments done in this laboratory seem to show clearly that the inhibitory pathway from the cortex in the cat exerts its influence entirely in the latter manner.

Cobb, Bailey and Holtz (1917) showed that stimulation of the superior cerebellar peduncle in decerebrate cats caused ipsilateral inhibition of the rigidity as long as the red nuclei and dentate-rubral tracts were

uninjured. They, moreover, believed that the inhibition of decerebrate rigidity by stimulation of the superior vermis depended on spread of current to the underlying superior cerebellar peduncles. Camis (1923) confirmed the observation of Weed that the cerebellum formed a link in the inhibitory pathway. Sherrington (1900) found that division of both superior cerebellar peduncles and the cortico-pontine fibers produced decerebrate rigidity; a fact which lays stress on the cerebro-cerebellar connections as a factor in the postural reflex.

Hughlings Jackson (1898) described the interaction of cerebral and cerebellar centers as follows, "Speaking very roughly the cerebellum represents movements of the skeletal muscles in order, trunk, leg and arm preponderatingly extensor-wise; the cerebrum represents movements of the same muscles in order, arm, leg and trunk preponderatingly flexor-wise. It is also supposed that impulses from motor centers of the higher levels of each sub-system continually act upon the motor centers of the lowest level; that impulses from each set of higher levels antagonize or inhibit each other in different degrees upon the different lowest motor centers; that the degree in which the cerebral and cerebellar influences antagonize each other is the same as the order of the degree of their different representation of movements of the muscles of the body."

Information concerning the course of the inhibitory path already suggested may be obtained by a study of hemi-decerebrate preparations. Unfortunately this evidence at present seems conflicting. Sherrington (1897–98) prepared cats with the fore-brain removed only on one side. If one hemisphere was destroyed the rigidity appeared, though with not the same certainty as after double ablation, chiefly on the same side as the hemisphere removed. The monkey, when suspended after removal of the right hemisphere, exhibited the following attitude. The right legs were extended in the pose characteristic of decerebrate rigidity. The tail was strongly incurved toward the right; that is, the convexity was toward the right and the tip was also toward the right. It resisted passive movement to the left and if displaced in this direction immediately on being released, flew back. The head was also pulled toward the right and retracted. The left fore-limb was distinctly more flexed than would be expected in the paralyzed condition of the animal, the left knee likewise. The same results were seen in the cat. The contrast between the attitude of the crossed and the ipsilateral sides was very striking.

Ipsilateral extensor rigidity consequent on removal of one hemisphere

was, however, neither so constant of production nor so persistent when it appeared, as the rigidity following bilateral ablation. After coming on it might totally subside and again reappear and so on several times over. There seemed under these circumstances a struggle between two conflicting influences as though a tonic influence from the still intact crossed hemisphere at times overcame and at times was overcome by another opposed influence from a lower center. Some amount of extensor rigidity on the side opposite to the lesion was not uncommon. In these hemi-decerebrate animals Sherrington studied the depressor effect of stimulating the motor cortex of the intact hemisphere as has already been mentioned.

Thiele (1905) also removed the fore-brain unilaterally. He found that when the section was far forward in the posterior portion of the thalamus the rigidity was contralateral but when the cut was made more caudad in the mid-brain the resulting rigidity was ipsilateral. This more posterior section might interrupt important mid-brain tracts, the rubro-spinal and the decussating fibers of the superior peduncles of the cerebellum.

Bazett and Penfield (1922) prepared a number of chronic hemi-decerebrate preparations (cats); the operation was always performed upon the right side. Two types of removal were distinguished. In one, the cut was made above the inferior colliculus leaving a varying part of the superior colliculus intact and most of the mid-brain ventrally. The red nucleus was not injured. In the other, the cut was posterior to the inferior colliculus to the anterior margin of the pons ventrally and the red nucleus was removed. The cats usually held the head to the left at the close of the operation and in every case eventually. The displacement consisted in retraction of the neck and a carrying of the head to the left away from the side of removal. The preparations that were able to walk fell to the left or walked in a circle to the left. In all of the anterior sections the animals turned their heads first to the right on recovery from the anesthetic; within one to five hours this movement was reversed and turning to the left appeared. The short duration of the preliminary turning to the right suggested that some structure was spared which was stimulated by traumatic irritation but later became paralyzed.

After the more posterior section, extensor rigidity appeared in the contralateral legs and flexor rigidity in the ipsilateral (Bazett and Penfield). After more anterior hemi-decerebration the pattern was tonic extension of the limbs on one side and tonic flexion in those of the other.

In certain cases the extensor rigidity appeared first on the ipsilateral side. This was, however, shortly reversed. In case of unilateral decerebration, after all excitatory effect had passed off, the control was removed from some structure whose unantagonized action produced extensor rigidity in the contralateral limbs and tonic flexor rigidity on the ipsilateral side. When the decerebration was bilateral, the flexor element usually disappeared and was superseded by extensor rigidity on both sides. But even after complete decerebration, tonic flexion occasionally replaced tonic extension.

As regard the laterality of the cerebral control, then, Sherrington (1897–98) maintained that after hemi-decerebration (level of section not stated) there was extensor rigidity in the ipsilateral extremities. Thiele (1905) found that hemi-decerebration at the posterior border of the optic thalamus produced contralateral rigidity, hemi-decerebration further caudad in the mid-brain ipsilateral rigidity. Weed (1914) and Cobb, Bailey and Holtz (1917) stimulated fibers in the mesial fraction of the cerebral peduncles which caused ipsilateral inhibition of the rigidity. Since the inhibitory control of postural reflex is bound up so closely with the mid-brain and since inhibitory paths probably decussate in this region at least twice (decussation of the fibers of the superior cerebellar peduncles and decussation of the rubro-spinal tracts) the discrepancy of laterality of the rigidity might be ascribed to the level of the section, above or in the mid-brain as was suggested by Thiele (1905). After removal of the area frontalis or of one cerebral cortex anterior to the optic thalamus contralateral extensor hypertonus results (Warner and Olmsted, 1923; Olmsted and Logan, 1925 and King, 1927). But on the other hand Weed (1914) traced a pathway in the anterior portion of the corona radiata and mesial portion of the crura stimulation of which gave ipsilateral inhibition of the extensor rigidity.

Moreover, Bazett and Penfield (1922) found that after hemi-decerebration just caudad to the inferior colliculus, extensor rigidity was present on the contralateral side. If the section was more anterior in the mid-brain the extensor tonus might at first be ipsilateral but eventually was always contralateral. In the more posterior section the red nucleus was removed and all decussating rubro-spinal fibers must have been severed. The only intact rubro-spinal fibers would be a few uncrossed fibers on the uninjured side of the brain. To those Weed (1914) attributed the small amount of contralateral rigidity remaining after removal of half of the mid-brain. But if Rademaker's theory is

correct that the function of the red nucleus is entirely inhibitory one would expect the extensor hyper-tonus (after removal of half of the mid-brain) to be greater ipsilaterally since no red nucleus fibers descend on that side of the cord.

Let us suppose that if possible, hemi-decerebration were performed in such a way that one red nucleus were removed and the decussation of the rubro-spinal fibers uninjured. Graham Brown (1913) and Weed (1914) have suggested that electrical stimulation of one red nucleus produces contralateral extension at the elbow and ipsilateral flexion at the elbow. One would expect that the unantagonized action of the intact red nucleus would produce a similar pattern of contralateral extension and ipsilateral flexion or extension on the side of the lesion, flexion contralateral to the lesion. But the experiments of Bazett and Penfield (1922) might be considered to demonstrate that just the opposite is the case.

A complete functional pathway involving the cerebral cortex, cerebellum and red nucleus has been shown to have a part in the control of the postural reflex. There are, no doubt, many other inhibiting factors about which we know at the present time very little. Thus Wilson (1924 and 1925) suggested that the function of the corpora striata was one of tone control and steadiness of innervation. Thiele (1905) believed that the optic thalamus had an inhibitory effect upon postural tonus. As Sherrington suggested, afferent impulses from the periphery have both a pressor and depressor effect upon the extensor tonus and it is probable that in the course of phylogenetic development the control of definite postural reflexes has been assumed by nuclei at different levels in the central nervous system.

SUBSTANTIATIVE STUDIES OF POSTURAL REFLEX. Sherrington (1897–98), Thiele (1905) and Bazett and Penfield (1922) all observed that after hemi-decerebration extensor hyper-tonus could be demonstrated in the extremities of one side of the body; on the opposite side the limbs often showed increased flexor tone. The presence of flexor hyper-tonus after experimental transection of the brain-stem has been further elucidated by the study of Richter and Bartemeier (1926) dealing with decerebrate rigidity in the sloth. The sloth normally hangs, back downward, from the branches of trees and its behavior in this posture is characterized by marked inactivity occasionally interrupted by slow deliberate movements. When on the ground it is scarcely able to raise its body on the legs and has to drag itself along.

The decerebrate sloth showed flexor rigidity of the limbs, claws and

trunk and reflex hanging in keeping with its normal posture (Richter and Bartemeier). The posture of the decerebrate animal varied with the level of the transection. When the red nuclei were removed the posture closely reproduced the animal's resting posture; the rigidity was marked in the trunk and limbs but was not as strong in the claws. For this reason they were not able to hang well. When the cut was made cephalad to the red nuclei, the rigidity of the limbs and trunk was less marked but the ability to hang was much greater. This flexor rigidity showed in common with extensor rigidity the lengthening and shortening responses, reflex inhibition from phasic reflexes and lack of fatigue. The dissimilarities had to do with spastic quality. For a certain range of movement the limbs did not exhibit any rigidity. In the fore-limbs the rigidity did not manifest itself until the lower part of the limb was nearly at right angles to the upper part; then it appeared very suddenly and in full strength. Richter and Bartemeier call this the "right angle flexor phenomenon."

After removal of a portion of the cerebral cortex, under favorable circumstances extensor hyper-tonus may be demonstrated but this is often masked by spontaneous alternate progressive movements and the animal is able to right itself and to walk. After transection of the brain-stem posterior to the thalamus, the extensor rigidity is more profound and progressive movements no longer occur. It was pointed out early in this paper that certain investigators (Thiele, 1905; Mella, 1923, and Laughton, 1924) have taken this to mean that in the region of the thalamus there is a center controlling progressive movements of the extremities. But it must be remembered that in the bulbar or spinal animal coördinated progressive beats of the extremities occur. A more logical explanation of the immobility of the decerebrate preparation might be that further inhibitory centers of postural control had been cut away and with the more profound extensor tonus, flexor reflexes seldom break through; as Sherrington says, "reflex standing engages the decerebrate preparation." The same explanation may also apply to the findings of Magnus and Rademaker (1924) that after section through the mid-brain important righting reflexes are lost. This is further suggested by experiments of the author upon the development of the postural reflex in young opossums (Langworthy, 1925). The strong enduring extensor hypertonus is apparently the factor that prevents the animal from assuming the erect posture and balancing the weight of the body on the legs. Woodworth and Sherrington (1904) found that progressive movements could be elicited from decerebrate

cats with maximal stimulation but they were of short duration. Bazett and Penfield (1922) from their study of chronic preparations further amplified the pseudo-affective reactions of decerebrate animals.

Studies of the postural reflex have advanced along other lines; certain of this work must now be mentioned briefly. Walshe (1922b and 1923b and c) reported a case of what he believed was complete decerebrate rigidity in man in whom a supra-cellar cyst pressed against the mid-brain apparently forming the equivalent of an experimental transection at that level. The arms were held lying across the body, semi-flexed at the elbow with fore-arms slightly pronated and wrist and fingers flexed. The legs were extended and adducted. The rigidity was plastic; the tendon reflexes were brisk and showed tonic prolongation. Walshe assumed that the flexion of the arms in human cases demonstrated that with the assumption of the erect posture the fore-limbs were freed from postural control. But recent experiments of King (1927) permit a different interpretation. If a cat, with the area frontalis removed upon both sides, is stretched out upon the floor on its back the hind-legs are greatly extended but the fore-legs are flexed, adducted and pronated. A tumor in the region of the hypophysis would injure first the fronto-pontine fibers, and it seems possible that Walshe's cases showed symptoms not of complete decerebration but rather of interruption of the cerebro-ponto-cerebellar control. Magnus and de Kleijn (1912 and 1915), Wilson (1920), Riddoch and Buzzard (1921), Stewart (1921), Meyers (1922) and Davis (1925) discussed other human cases. Walshe (1923a) described tonic or postural reflexes that could sometimes be elicited in hemiplegic patients.

Hunter (1924) attempted to show that the postural tone of skeletal muscle was controlled by the sympathetic nervous system. His work stimulated many observations but has not been confirmed by a majority of investigators. Coman (1926) gave a rather complete survey of the current literature on this subject. He further demonstrated that after cutting the ventral roots to one of the forelegs in the cat no tonus was present after decerebration even though the sympathetic reflex arc was uninjured.

Comparative studies of postural reflex. The postural reflex has been little studied from the comparative viewpoint; there has really been no true systematic attack on the problem. In lower mammals Rogers (1923 and 1924) and Weed and Langworthy (1925) studied the reactions of adult opossums after transection of the mid-brain. Decerebration was followed by the development of a true rigidity,

involving the extensor musculature of neck, trunk, tail, fore-legs and to a lesser extent of hind-legs. The preparations differed from the higher mammals under similar experimental conditions by exhibiting very frequently rhythmic, well coördinated movements of progression.

The brain of the alligator, a reptile, was transected at different levels and the effect upon posture studied (Bagley and Langworthy, 1926). Removal of the fore-brain was without any apparent effect but if the mid-brain was also removed, abnormalities in locomotion occurred. With the first few steps the legs became more and more extended till the extension in the fore-legs was maximal. This extension seemed to inhibit further progression and the animal stood quiet, high upon its legs. Upon strong stimulation the alligator attempted to walk but lost its balance with the first step and fell to the side. When at rest the legs were flexed in a normal manner. No resistance to passive movements of the extremities was ever noted.

Similar phenomena may be demonstrated in the pigeon. Rogers (1922) in his study of the behavior of pigeons after removel of portions of the brain gave little information concerning changes in postural control. Hunter (1924a and b) published the results of removal of the corpus striatum upon the normal posture of birds, the sea-gull and domestic fowl. Langworthy (1926b) found abnormalities in postural control after removal of the corpus striatum of the pigeon. If the mid-brain be then removed, together with the optic lobes, the legs are held flexed close to the body. But, if now the claws are held upon the table and an attempt made to balance the body weight upon the extremities, the legs arrive by a series of jerks at a maximally extended position; so marked indeed that it is impossible for the bird to stand and it falls backward. The extension is lost as soon as the animal falls. No extension hyper-tonus could be demonstrated when the birds were quiet and undisturbed.

Developmental studies of the maturation of the postural reflex in young animals have been made. After decerebration half of the animals in a series of new-born and young kittens did not show the extensor hyper-tonus evident in adults but on the contrary become enormously active (Weed, 1917). If suspended in a warm-box, alternate progressive movements of all four legs continued for considerable periods of time. When placed upon the floor they sprawled along at a rate slightly faster than that of normal animals of the same age. The postural factor had not yet developed while the tendency toward progression seemed to be increased by the operation. Rigidity developed in the older prepara-

tions as evidenced first by extension and adduction of the fore-legs when at rest, later by a slight resistance to passive movement of the extremities. By the fourth week of life, the postural reactions approached those of the adult. As rigidity came to be dominant, the progressive tendencies were inhibited; during the transition period, the animals tended to show postural tonus at the beginning of the experiment and to be more active later. Graham Brown (1915b) previously had decerebrated one litter of fetal kittens, all of which showed alternate progressive movements without rigidity.

Continuing this work on other species it was shown that the reactions of new-born animals after decerebration depended to a great extent upon their maturity at birth (Langworthy, 1924). Thus rabbits, born very immature after a short gestation period, were very active and the postural factor did not become dominant for some weeks. Guinea pigs on the other hand, born with the eyes open, possessing a well developed coat of fur and able to run about almost as actively as adults, never exhibited active progressive movements after decerebration but all developed rigidity. The gait of the decerebrated young of these species corresponded with the normal mode of progression of the adult. Thus the kittens usually displayed alternate progressive movements, trotting, seldom galloping; the rabbits on the other hand hopped and seldom trotted. Later the brain-stems and spinal cords of the animals studied in these two series of experiments were stained by the Weigert-Pal method and the progressive myelinization studied (Langworthy, 1926a). In the young animals (kittens and rabbit) in which decerebration was followed by movements of prolonged progression, myelinization in the lower portion of the brain-stem and spinal cord was not yet complete. In more mature animals (guinea pig, kitten and rabbit) in which decerebration was followed by extensor hyper-tonus, myelinization of the long tracts was far more advanced. A correlation between the myelinization of the rubro-spinal tract and the appearance of extensor hyper-tonus was strongly indicated.

Findings of Laughton (1926) differ markedly from the physiological observations reported above. He found that in very young kittens, rabbits and puppies there was marked rigidity in the fore-legs but no rigidity in the hind-legs. Variations in technique may explain these divergencies in experimental results.

Similar studies of the development of both progression and posture in young opossums were later reported (Weed and Langworthy, 1925; Langworthy, 1925). The opossum young are born after a very short

gestation period when only about 11 mm. in length. Though the fore-legs are well developed the hind-legs are merely extensive buds. These vigorous, air-breathing embryos and fetuses may be detached from the nipple in the pouch of the mother for observation and study. Pouch-young opossums of less than fifty days of age showed the same reflex and progressive activities after decerebration as in the normal state but these activities were of lesser degree. Animals of slightly older age (about 56 days) showed, after decerebration, outspoken tendency to unceasing progression of all four legs more marked than before the transection. And in a still older group (pouch-young of about 64 days), prolonged progression on maximally extended legs characterized the decerebrate state. In the oldest group of pouch-young opossums (about 54 days), the extensor out-thrust of true decerebrate rigidity appeared after the transection of the neuraxis, the progressive tendency being thereby suppressed.

Tilney and Casamajor (1924) have attempted to correlate the activity of new-born and young kittens with the development of functional pathways in the central nervous system. From their study they are convinced that the long tracts become functional at the time they receive their myelin sheaths. For example, the righting reflex is operative at birth. At that time well myelinated vestibulo-spinal fibers can be seen. It is also interesting to observe that although the vestibulo-spinal tract is myelinated the postural reflex is not operative in new-born kittens.

CONCLUSION

Certain important general considerations concerning the cerebral control of the postural reflex arise from a study of these conflicting experimental data. First, it seems probable that the predominant afferent impulses responsible for the production and control of postural tone are proprioceptive from the muscles of the extremities. Several recent investigators have overlooked the fundamental importance of these connections in the attempt to elucidate the rôle played by other afferent impulses in the modification of postural patterns.

The tracts mediating proprioceptive impulses in their cephalic course are constituted of large numbers of fibers distributed to the vermis of the cerebellum by the dorsal and ventral spino-cerebellar tracts of Flechsig and Gower, and by fibers arising from the nuclei of the posterior columns (gracilis and cuneatus). The termination in the cerebellum of this large group of fibers carrying afferent stimuli from muscles

in itself suggests the importance of this organ in the control of posture. On this basis, the vermis may theoretically send excitatory or inhibitory impulses to the final common path by at least three efferent systems (reticulo-spinal, vestibulo-spinal and rubro-spinal). The remaining fibers mediating proprioceptive sensitivity travel rostralwards in the medial lemniscus to the thalamus and finally to the cerebral cortex. They give off collaterals to many important structures in the brain stem and thus make reflex connections of all grades of complexity.

The efferent pathways from the brain that produce and control postural tone can likewise be discussed at the present time only in broad general terms. Here Sherrington's exposition of the final common path forms an ideal starting point for theoretical considerations. It is obvious that cells (somatic motor and autonomic) in the anterior horn of the spinal cord and in certain nuclei of the cranial nerves send fibers outside of the central nervous system to terminate upon striated muscle and thus form the only pathway by which impulses from the brain and spinal cord can influence the tone and contraction of this muscle. In the present study of postural tone in striated muscle we may ignore the autonomic component of the final common path for although autonomic nerve fibers apparently end upon striated muscle their functional importance is not yet understood, and it would appear from recent experiments that they have no part in the production of tone. Around the somatic motor cells of the final common path several tracts of fibers from the brain end. It might at first be thought that the impulses carried along each of these efferent pathways have a widely different influence upon the tone and contraction of muscle. But although an individual tract may be distributed predominantly either to flexor or extensor centers, nevertheless all these tracts must finally exert their control through the neurones of the final common path. The sum-total of the activity of all the efferent cerebral tracts therefore must be considered as a unit, for it tends to increase or decrease the activity of the cells of the final common path.

A more correct interpretation of the function of these different efferent cerebral pathways will be obtained if their phylogenetic development is kept clearly in mind. From time to time under the stimulus of increased peripheral or central connections, portions of the brain have undergone enormous increase in size and from these areas new efferent pathways have developed, discharging their impulses either directly or indirectly around the cells of the final common path. In phylogeny now one efferent pathway and now another has assumed a dominant

control over the tonic and clonic contractions of striated muscle. In the amphibia, local reflex arcs in the spinal cord are capable of controlling the postural reflex in a fairly normal manner. But in the ascending phylogenetic scale, the final common path has come to be more and more under the control of cerebral centers. The first important efferent pathway arose from scattered cells in the reticular formation of the brain stem: the vestibulo-spinal and then the rubro-spinal tracts developed. The efferent connections of the corpora striata show important phylogenetic variations as Wilson has pointed out. But in mammals these nuclei make only indirect connections with the final common path by way of the red nucleus, substantia nigra and thalamus. And finally, the cerebral motor centers have undergone progressive differentiation, exerting their influence either directly or through the cerebellum.

Studies of the postural reflex then must take into consideration the relative functional importance of these efferent pathways in the particular animal studied experimentally. Among many well-known examples one might mention the wide variations in behavior of man compared with the ordinary laboratory mammals after injury to the cortico-spinal tracts or after complete transection of the spinal cord. At the present time there is needed a correlation of comparative physiological studies of the central nervous system with comparative morphological knowledge. This information will gradually be forthcoming. And with such a comparative viewpoint many of the divergent results reported in this review become more comprehensible.

In the present paper the modern methods of study of the postural reflex and the viewpoints from which investigators approach this subject have been discussed. It is very clear that the evidence conflicts at many points and that the final word has not yet been said. Technical difficulties in the physiological investigations arise in general from two causes. First the pathways producing and those inhibiting or controlling the postural reflex may be both stimulated or injured by the same procedure, for example, by the severing of the superior peduncle of the cerebellum. And again the reflex arcs involved undoubtedly form long circuits which may be stimulated or broken at many points with the same equivocal results. Moreover, different investigators, each working on isolated phases of this same problem do not yet agree as to a definition of decerebrate rigidity. Decerebrate rigidity must, as has already been explained, include all the physiological abnormalities resulting from a transection of the brain stem at a definite level. It is also unfortunate that no satisfactory quantitative method of describing

extensor tonus is applicable to many of the experimental procedures. Almost all portions of the central nervous system appear to have a part in the nice control of the postural reflex and with the progressive development of the brain this control is constantly assumed by higher levels. The great interest in this subject in recent years suggests that further advances are to be expected in this field possibly with broad applications to the understanding of cerebral function.

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BONE MARROW

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I. THE VASCULAR SYSTEM. In the analysis of the structure of bone marrow, the most important factor is the nature of its vascular system. Here, in perhaps the last place of the animal organism, the old question of an open versus a closed circulation is still being studied. The matter is, however, clarified over the earlier discussions, inasmuch as we now know that most blood vessels are closed, and now have adequate physiological criteria for the demonstration of an open circulation, as exemplified in the spleen. These criteria are the presence of red blood cells in tissue spaces physiologically, which has a corollary in the invariable occurrence of extravasations in experimental injections, and the presence of a specific muscular mechanism, such as the muscles on the trabeculae of the spleen, for the return of the red cells to the blood stream. These two factors are the essential evidences of an open circulation. A modification of this form has been described by Woollard and Wislocki (1) for hemal glands, in which the red blood cells pass from the vessels into blood spaces, from which, however, there is no pathway and no mechanism for their return into the circulation, so that they degenerate locally instead.

The question of open or closed vessels is important to the understanding of the formation of red blood cells. The entire physiological function of the red cells is within vessels, with the possible exception of the spleen; the concept that they also arise within vessels correlates with the idea of a closed system. For the white cells, the matter is not so significant, since it is generally accepted that they arise outside the vessels, and that they both enter and leave any part of the vascular system by means of their own motility.

The nature of the circulation of the bone marrow has recently been reinvestigated by Doan (2), (3). He has shown that the key to the analysis of the vessels of the marrow is to be found in an experimental reduction to a simplified state. By underfeeding, he was able to reduce the red marrow of the radius of the pigeon to an extreme hypoplasia,

in which the parenchyma consisted of three elements only, blood vessels, fat cells, and a minimal residual framework of reticulum and reticular cells. The general pattern of the vascular system of bone marrow has long been known: it is made by the main nutrient artery with accompanying veins, passing toward either epiphysis and its capillary branches to the venous sinusoids, together with numerous anastomoses of this system with the small vessels of the bone along the shaft and with larger epiphyseal vessels in the mature bone.

Doan demonstrated that the circulation of the avian marrow is closed. He discovered that there is a vastly greater bed of venous sinuses in the marrow than can be patent to the circulation at any one time on account of the rigid confines of the marrow cavity, and that these sinuses, when collapsed, appear as capillaries which are so abundant as to pass between every one or two fat cells. These collapsed vessels can, however, be demonstrated by lines of granules of ink between the two endothelial walls by injections that have been pushed to the maximum without rupture in hypoplastic marrow. Doan analyzed the difference between the injection of these collapsed capillaries and the appearance of extravasation. The importance of the collapsed capillary in physiology was first shown by Krogh (4), (5), who demonstrated that the number of open capillaries in muscle is the register of the amount of function.

In the marrow, the capillaries that lead directly from the arteries of the third and fourth order from the nutrient arteries, making the "transition capillaries" of Doan, are not the functioning capillaries, as in other organs, but rather they lead into tufts of sinusoids (figs. 4, 5, 6 in the paper by Doan, 3). These sinusoids make the functioning vascular bed of the marrow; they have walls like capillaries and appear like them when collapsed, but when open they have the diameter and lumen of large veins. The nature of such vessels, having the size of veins but functioning as capillaries, was analyzed by Minot (6) in 1900, who introduced the term sinusoid. The sinusoids of the mesh which happen to be closed at any one time are called by Doan "intersinusoidal capillaries," and it is his concept that there is frequent interchange, a given vessel serving for a time as a collapsed capillary and then as a sinus wide open to the circulation. In other words, in the functioning bed of marrow, a large part of the vessels must always be collapsed in the space available, while a part are at the same time so widely dilated as to make a zone of sluggish blood flow. The sinusoids which are closed to the circulation at any one time are the actual or the potential site of the

formation of the red cells. These collapsed vessels thus become as significant an index of function as the closed vessels of other organs, though in a different and more complex way, since in the marrow the formation of the red cells has to be maintained as well as their circulation. Doan also showed that the number of capillaries in the collapsed phase is greatly increased in gelatinous marrow in association with erythropoiesis and that the collapsed capillaries connect by conical openings with the sinusoids, a point of significance in connection with the method of delivery of the red cells into the circulation. Thus the vascular system of the marrow consists of arteries running longitudinally in the center, and transition capillaries leading to sinusoids, which correspond to the functioning capillaries of other organs. These run transversely. The sinusoids are in part patent to the circulation, in part collapsed and erythrogenic in function. Finally, the sinusoids lead into veins which accompany the arteries.

From the start, the question of an open or a closed circulation for avian marrow was easier to determine than for mammalian forms. In 1881, Bizzozero and Torre (7) described the circulation of the marrow of the bird as a closed type on the basis of the histological structure of the walls of the sinuses and capillaries. They were the first to find that the marrow of the bird could be simplified by underfeeding and that in preparations of such marrow it could be made out histologically that the vessels had intact walls. A closed circulation for avian marrow was confirmed by Denys (8) and van der Stricht (9). Denys (1888) showed that the reason for the difficulty of injecting the vessels of marrow lies in the fact that the circulation is structurally adapted to slow the blood flow; thus he found that a pressure which brings out all the vessels of muscle, fills only the main arteries in marrow. At higher pressure, he found that the sinuses became filled, but the capillaries remained empty, and thus he postulated that there were two types of vessels, one (the sinusoids) that carried blood, and the other (the intersinusoidal capillaries) that carried only plasma.

Concerning the type of circulation in mammalian marrow, there has been less agreement. Of the earlier discussions, the most interesting accounts are those by Bizzozero (10) and Neumann (11), both written in 1869. This was a significant period. The year before, Neumann (12) had announced in one paragraph that red cells arise in marrow, and then both Bizzozero (13) and Neumann (14) found that not only red but also white blood cells are formed there. Shortly before (1862), von Recklinghausen (15) had found that the reaction of silver nitrate

demonstrated a cellular lining, endothelium, for lymphatics, and Hoyer (16), in 1865, had applied the method to blood vessels and found their endothelium. Hoyer (17) was, however, unable to silver the sinusoids of the bone marrow, and hence postulated a wide open circulation there. Bizzozero (10) discusses these observations with which he differed, since he could find the endothelial lining for the capillaries and could inject the veins through an interstitial puncture. He saw a distinct border for the sinusoids in many places, and inclined toward the view of a closed circulation, but judged that a satisfactory demonstration of it needed further work. He gave an excellent description of the vascular pattern in the marrow of the rabbit. He described the arteries with their branches coming off at an acute angle, and found that the third and fourth orders lead to the capillaries, and then found two systems of veins, the sinusoids which he terms secondary veins, with their radiating pattern across the marrow, and the true veins accompanying the arteries. He found the capillaries by studying gelatinous marrow. Thus he made many of the observations which have been demonstrated in recent studies, but lacked a concept of the nature of the sinusoid and did not know that the capillaries which he saw in gelatinous marrow could, under other circumstances, be patent sinuses.

Neumann (11) came even closer to the true relation of artery to sinusoid and sinusoid to capillary. He described the arteries as becoming reduced, while still preserving their tunics, to the size of a capillary and then emptying abruptly into large capillaries (the sinusoids). He saw this as a mechanism for slowing the blood flow, and thus had the concept of sinusoidal circulation. He described these capillaries (the sinusoids) as having a diameter six times as wide as an ordinary capillary, and as being lined by a thin membrane. Finally, he said that at frequent intervals there were little diverticula along the capillary walls from which extended, thread-like, processes or sprouts exactly like the vascular sprouts or *anlagen* which had recently been described for the vessels of the tadpole's tail. As far as we have found, Neumann is the only one who observed these conical openings of the intersinusoidal capillaries, before they were described and interpreted by Doan. In 1877, Langer (18) published a paper in favor of the concept of a closed circulation in mammalian bone marrow. In spite of these observations, Rindfleisch (19), van der Stricht (9), and Muir (20) favored a return to Hoyer's concept of an open circulation.

Van der Stricht (9), in 1892, recognized the significance of the nature of the vessels in marrow to the subject of the origin of red blood cells,

and thought that the vascular system and the method of formation of the red cells differed in birds and mammals. In birds he found the circulation to be closed, and that erythrogenesis was intravascular; in the rabbit, on the other hand, he thought that the circulation was wide open so that the blood, the mature red cells, wandered freely in the meshes of the connective tissue. Thus he regarded the formation of red cells as extravascular in mammals. A modification of this concept, in connection with the bone marrow of the mammal, is to be found in the work of Maximow (21), who thought that the red cells formed in close contact with the outer wall of the vessels and that from time to time there were local breaks in the continuity of the endothelium which allowed the plasma and the mature red cells to flow out into masses of maturing but still nucleated red cells.

It is now clear that the question of an open or closed circulation in bone marrow concerns the nature of the walls of the sinusoids which represent that part of the capillary bed which is patent to the circulation at any one time; the point at issue is whether they have intact or fenestrated walls. The extreme thinness of the walls of these sinusoids, the variations in diameter of their lumen, and the dense packing of cells along their borders, together with phenomena associated with the opening of hematopoietic capillaries to be discussed later, have constituted the difficulty of this problem.

The whole question of the circulation of mammalian bone marrow has been thoroughly restudied recently by Drinker, Drinker and Lund (22), and by Doan, Cunningham and Sabin (23). Drinker and his co-workers undertook the study of the method of delivery of the red cells into the circulation in dogs, cats, and rabbits. They developed a carefully controlled method for the perfusion of the marrow with hirudinized blood and with physiological salt solution, and followed the delivery of the red cells into the perfusate. In the animals in which the function of the marrow was at its normal level, there was no increase in the number of nucleated cells put out from the marrow, even with prolonged perfusion; in animals with marrows markedly hyperplastic in the formation of red cells, there was an increase in nucleated red cells. They concluded that immature red cells, which proved to be impossible to dislodge except in the hyperplastic state, were not developing in the pathways of free flow of the circulation. At the close of each experiment, they put India ink into the perfusing solution for a record of the free pathways of the circulation. In these experiments they could show that the circulation in the marrow is through closed vessels. Moreover,

they repeated the experiments of Bunting (24) with endothelial toxins, ricin and saponin, and confirmed his observations of the production of hemorrhagic marrow. The same was also shown by Firket (25). The judgment that hemorrhage in bone marrow is a pathological condition is a recognition that a closed circulation is normal, for an open circulation means physiological hemorrhage.

The closed nature of the circulation in the marrow of mammals is also indicated in the work of Doan *et al.* (23) in injections of the marrow of rabbits from which the granulocytes had been removed by the injection of large doses of inactivated typhoid bacilli; in these preparations the evidence of intact vessels was convincing.

The question of an open circulation has, however, been revived by Jordan and Baker (26) for amphibian material. They have shown that erythropoiesis in the frog takes place largely in the spleen, except for a short period after hibernation, when the bone marrow becomes somewhat involved; but after splenectomy, there is a marked transference of erythropoiesis to the marrow, making favorable material for study. They made injections with a carefully controlled technique, introducing only a small amount of ink into the heart, while withdrawing a compensatory amount of blood from a vein and allowing the pressure from the heart beat to carry the injection mass into the marrow. It is now clear that with an open circulation the fenestrae in the walls of the vessels can be seen with the microscope, as was demonstrated by Mollier (27) for the ampullae of the splenic arteries and the walls of the veins (sinusoids) of the splenic pulp, and that from such vessels both plasma and cells flow out physiologically, as does water through a sieve; in other words, an animal bleeds into its own spleen and an open circulation is there plainly and unequivocally demonstrable at blood pressure. Jordan and Baker describe a variation of such an open structure for the walls of the sinusoids in amphibian marrow, namely, that the endothelium fades off into a reticulum, leaving no definite walls for the vascular channels, a condition which would call for a wide open circulation physiologically. Their figures of injections are, however, not convincing in that they are not comparable to those that are obtained by a single spurt of ink into the splenic artery at blood pressure and not comparable to hemorrhagic mammalian marrow. There is, of course, one striking contrast between conditions of spleen and bone marrow; the latter is not an extensile organ, so that it might be postulated that the fat cells could be packed in marrow so densely as to offer resistance to the spread of an extravasation; but in an organ that is non-extensile, it is difficult to imagine what mecha-

nism could bring about the return of the inert red cells to the circulation. To sum up the question of the nature of the circulation in bone marrow, the balance of evidence at the present time is toward a closed type. The significant points in the structure of the vascular system are that the circulation is sinusoidal, that is, adapted to a sluggish flow, and that a large part of the sinusoids at any one time are collapsed and erythropoietic in function.

II. Distribution of red, fatty and gelatinous marrow. The question of the distribution of red marrow is an important one and varies markedly in different animals. As is well known, in the larger mammals and in man, the marrow of the long bones of the adult is predominantly yellow, and thus there is a large reserve potentially available for the formation of blood cells, but not used under normal conditions. The best account of the distribution of red marrow in the human being is that of Piney (28). He found the bone marrow at birth to be of a pink rather than a red color; by the age of seven, there is some fat in the marrow; between twelve and fourteen years, there appears a definite macroscopic patch of fat in the middle of the shaft of the femur, which gradually extends until in the adult there is only one patch of red marrow left near the upper end of the diaphysis. The same process takes place in tibia and fibula, in humerus, radius, and ulna, only more rapidly and completely. The epiphyses also become entirely fatty, as do the bones of carpus and tarsus. Red marrow is to be found in the ribs, vertebrae, sternum, in the bones of the skull and in the os innominatum.

In human pathology this distribution is made use of by selecting for routine study a bone in which the formation of blood takes place normally, usually a vertebra, less often a rib, to determine any lowering of activity; and a long bone, usually the shaft of the femur, to detect any extension of hematopoietic territory. For human biopsies the center of the tibia (Ghedini, 29, 30; Morris and Falconer, 31; Caronia, 32; Kramár and Hensch, 33; Peabody, 34) or the sternum (Seyfarth, 35; Yamamoto, 36) is used. Kramár and Hensch have applied the technique to the infant. In the dog and the cat the long bones are occupied by red marrow but with spicules of bone. With rabbits and guinea pigs the cavity of the long bones is involved in blood formation, and since there are no spicules of bone, these animals afford excellent material for study, as the difficulties of decalcification may be avoided. In birds the humerus is filled with air for lightness, and, as shown by Meltzer (36A), for respiratory function as well; but the radius and ulna are normally filled with red marrow; spicules of bone are sometimes pres-

ent, but at times are entirely absent; in starvation, when the marrow of the radius has become entirely hypoplastic, there may be some compensatory increase in red marrow in the femora.

The significance of the fat in marrow, both the amount that is present in red marrow and in marrow that is normally wholly yellow, has long been known, but the rapidity with which the fat can be mobilized out of the marrow is only to be realized through experimental methods. In his experiments with induced hypoplasia in the pigeon, Doan (3) underfed a bird until it had lost 150 grams; he then removed a small bit of marrow, under ether, from the radius at biopsy, and found that it had been reduced to the extreme fatty form; then the animal was fed, and in twenty-four hours, as shown by a second biopsy from the same bone, there were marked changes in the fat cells, while in forty-eight hours, when the pigeon was killed, the fat has almost entirely disappeared and the marrow had become gelatinous. The process is as follows: the fat cells first return part way toward their embryonic state. In the development of a fat cell, the lipoid is first laid down around a centrally placed nucleus in small droplets. These gradually fuse into larger and larger droplets, until finally the entire content of fat is in one large sphere. In the regression of the fat cell, the first change is the splitting up of the large sphere of fat into small droplets; the nucleus rounds up as if released from pressure; the droplets of fat then start to pass out of the cell on the side toward a patent sinus, leaving branched processes of cytoplasm to mark this shrinkage. The meaning of these changes in the fat is that the cavity of the bones is entirely filled with tissue, excepting the pneumatic wing bones of birds, and when there is any increase of cells in marrow, something has to pass out to make room. For acute expansion there are only three things which can pass out, namely, the fat, the blood circulating in the vessels, and the newly developing marrow cells. The fat cells make the main regulatory mechanism for space available to new marrow cells when there is any marked change in the amount of formation of blood, and on this account the fat in marrow is in an extremely labile state. The fact that the entire fat can be demobilized from the bone of a bird in forty-eight hours is evidence of this. In the study of tubercular rabbits, Doan and I have seen large masses of fat droplets in fresh specimens of peripheral blood at a stage when a marked anemia and leucopenia were correlated with a massive development and encroachment by tubercles in the bone marrow. It has been shown by Bloor (37) that there is an increase in unsaturated fatty acids in the blood after hemorrhage. Thus the histo-

logical and chemical evidence is toward the view of a shifting of the fat from the marrow cavity, when increased formation of cells is demanded.

When the fat disappears so rapidly from the marrow, its place is not at once completely occupied by blood-forming tissue, which has no such speed of regeneration, but Doan has shown that its place is rather immediately occupied by a gelatinous substance which fills in all the spaces left by the shrunken fat cells. Thus, fasting gives a fatty marrow; feeding after this induced hypoplasia gives the following sequence of events: removal of fat, gelatinous marrow, erythropoiesis.

Gelatinous marrow has long been known. It was described by Bichat (38) in 1801, who saw it in certain diseases, for example in rickets, and it is well known to pathologists. Neumann (11) and Bizzozero (10) both described gelatinous marrow in 1869, and the latter noted that it often occurred in young animals and was often red in color. In 1881 it was shown by Bizzozero and Torre (7) that it could be produced in birds by underfeeding; while in 1904, Jackson (39) repeated these experiments for the pigeon and demonstrated the same for the rabbit. Its frequent occurrence under pathological conditions has led to the universal view that it is a form of degeneration. Neumann (40) described it as a transformation into a myxomatous tissue in which the fat cells become changed into branching mucoid cells, lying in a homogeneous matrix. Muir (41) noted again that gelatinous marrow might have a red color, which was confirmed by Dickson (42). Muir found that the gelatinous substance is an albuminoid and believed that it came in part from the fat. Dickson regarded the gelatinous stage as a form of degeneration, but he noted that in gelatinous areas the vascular channels often remained patent to circulation to a much greater extent than might be expected in areas of degeneration. These observations of Bizzozero, Muir, and Dickson can now be interpreted in the light of Doan's observations that erythropoiesis begins in the gelatinous marrow after hypoplasia. Stefko (43) recently had an opportunity to study the marrow from sixty-three human cases of starvation and found gelatinous marrow as characteristic. The source and the nature of the gelatinous substance are not, as yet, known.

Beside the mechanism for shifting fat out of marrow, there is another means of increasing space which can be used under extreme need. Drinker *et al.* (22) found that there is a decrease in the spicules of bone in the marrow of the tibia of dogs after repeated hemorrhage; and Johnstone (44) has described an enlargement of the marrow cavity through an actual thinning of the compact bone of the border of the ribs in the dog during a phase of increased formation of red cells after splenectomy.

It is clear that both the wholesale removal of the fat and even more the removal of bone represent mechanisms in conditions that call for a change in the level of blood formation from the normal. The physiological shifting out from the marrow of each successive generation of mature red cells and white cells in the normal function of the organ is, on the other hand, more probably compensated for by vasomotor regulation.

Yellow marrow is at all times a potential reserve for blood formation; there is some evidence that it may also be a special factor of safety in infections. Infection of red marrow more often than of yellow was indicated in typhoid fever by Quincke and Stühlen (45) and Fränkel (46), (47); Müller (48), in a series of cases of influenza, found that the red marrow had been invaded by organisms and damaged enough to cut down granulopoiesis, while the yellow marrow had escaped the infection and was becoming active. Hartwich (49), (50) has not found that yellow marrow infected less often than the red; but Müller (51) reported the vertebral marrow involved in over 80 per cent of a series of different infections and the marrow of the long bones in 25 per cent. Thus, though the evidence is conflicting, the structure of yellow marrow with its high proportion of collapsed capillaries might be adapted to give a smaller dose of organisms per unit area.

III. Formation of red blood cells in marrow. The structure of marrow with relation to the formation of blood cells was not analyzed in the active state, for so crowded are the cells that their relationships are difficult to determine. To become familiar with the process, it was necessary to begin the study of the formation of blood cells in the early stages of recovery from the hypoplastic state, and thus to determine methods to make marrow inactive and then to stimulate it at will.

Erythropoiesis in avian marrow. The crowding of cells is far less in avian marrow than in mammalian, and hence the general agreement that erythropoiesis takes place within the vessels in the bird (Bizzozero, 52; van der Stricht, 9; Muir, 40; Danchakoff, 53). The meaning of this intravascular origin of the red cells in the bird was made clear by the observations of Sabin (54), in studies on the living chick blastoderm of the second and third days of incubation: this study proved that red cells arise within vessels because they come from endothelium, or that the power to synthesize hemoglobin develops in the angioblast and its derivative, endothelium. It was then shown by Doan (2), (3), (23) that endothelium is as fundamental to the formation of red cells in the adult bird as during the stages of incubation. As has been described, after the

period of underfeeding the marrow was fatty, and forty-eight hours after feeding was begun the fat had been removed and the marrow had become gelatinous.

The next phase in regeneration, after the gelatinous state, was erythropoiesis alone, so that there was a chance for the study of this process completely separated from granulopoiesis. In the gelatinous marrow the dense capillary plexus was obvious both on account of the closing of many of the patent sinuses, as contrasted with the normal state, and because of the metaplasia and swelling of the endothelium. There is an important physiological condition to be considered when so large a proportion of the sinusoids is collapsed, because the entire marrow has then a low blood supply, or is then in a state of low oxygen tension. This point is of great significance for erythropoiesis. It is in agreement with the observations of the increased formation of red cells in high altitudes (Bert, 55; Viault, 56; Douglas *et al.*, 57; Barcroft *et al.*, 58;) and with the work of Brill (59) on low oxygen as a stimulus to marrow, and of Dallwig, Kolls and Loevenhart (60) on increased erythropoiesis in animals kept in an atmosphere of low oxygen tension. Jordan and Speidel (61) have shown the same thing in amphibian material, expressing it in terms of the stimulus of CO_2. Campbell (62), (63) has shown not only that low oxygen tension increases the formation of red cells, but that high oxygen decreases their formation. Moreover, during their increase there is a change in the color index.

It should be noted that the point that low oxygen tension favors the formation of red cells is not contradicted by the well known fact of erythrogenesis in the spleen, inasmuch as the blood of the spleen is predominantly venous and relatively stagnant, as compared with the circulation in other organs. It is clear that the conditions in the spleen are adapted to the formation of both red and white cells; the sluggish blood flow gives low oxygen tension for the red cells and brings the maturation factors in right concentration for the white cells.

The first step in the formation of red cells is the swelling of some of the endothelial cells of the capillaries; then follows the division of the swollen endothelial cells. We have now been able to see that this swelling or phase of growth actually precedes division, by watching the process in the vessels of living chick blastoderms growing in a solution of liver extract, for which we are indebted to Dr. George Minot and Dr. Edwin Cohn.

In avian marrow, division of these enlarged endothelial cells was found with the equator of the spindle parallel to the wall of the vessel; when

the equator of the spindle is perpendicular to the wall of the vessel, two endothelial cells result; when parallel to the wall, the outer cells become the endothelial wall and the inner ones megaloblasts.

In the next stage after the swelling of the endothelial cells of the intersinusoidal capillaries, the capillaries become even more prominent in the depleted marrows by the development of lines of megaloblasts within them. The megaloblast is the first stage in the maturation of the red blood cell. It is readily discriminated in sections by the large vesicular nucleus, the characteristic large nucleolus, and the scanty chromatin; the amount of hemoglobin in the megaloblast is so slight that it cannot be seen at all when the cell has been stained in any basic dye, since the basophilia of the cytoplasm is so strong that it obscures the weak acidophilia contributed by the hemoglobin. In fresh preparations, on the other hand, a minimal amount of hemoglobin can be detected and the cell has large numbers of mitochondria. It is discriminated also by an extreme grade of the phenomenon of reticulation. (These supravital reactions are shown in figures 44 and 58, plate IV, in article by Doan *et al.* (23)). The origin of megaloblasts from endothelium of the intersinusoidal capillaries in bone marrow of pigeons at different periods of regeneration has been confirmed by Muller (64).

As erythropoiesis proceeds, the megaloblasts give rise to early erythroblasts; then late erythroblasts develop, and finally the nucleated red cells of the adult avian type (Sugiyama, 65). In the bone marrow the megaloblasts remain against the endothelial wall and the older types are thus to be found in the center of the capillaries which dilate to meet the needs of the increasing number of cells (fig. 6, in Doan *et al.*, 23). This arrangement was illustrated by Denys (8) in 1877 and they by van der Stricht (fig. 50 on plate X of van der Stricht's paper, 9), who introduced the term of hematopoietic capillaries for such vessels.

Erythropoiesis in the mammal. In spite of the complexity of mammalian marrow, due to the crowding of the cells, the same fundamental structure can be determined. In the mammal also the red cells arise within vessels because they come from endothelium (Doan *et al.*, 23), and the white cells arise in the intervascular spaces. The methods for depleting and stimulating mammalian marrow are more complex and the study of the control by diet has only begun (Whipple *et al.*, 66; Robscheit-Robbins *et al.*, 67; Murphy and Minot, 68; Minot and Murphy, 69).

In 1901 and 1902, Muir (41), (70), who had found that the red cells of avian marrow develop within vessels, observed that a separation of the formation of red and white cells could also be seen in mammalian

marrow in areas in which red marrow was extending. He described the white cells beginning near patent sinuses and nucleated red cells first appearing in lines or small clumps. The concept of erythrogenic and leucogenic centers in mammalian marrow was still more clearly formulated by Selling (71), (72), who saw that after poisoning with benzol the regeneration involved two types of islands, erythroblastic and granuloblastic, and that each remained separate and was made up of one strain for some time. Then Bunting (24) found that though it might be difficult to demonstrate the two types of centers as distinct in active marrow, nevertheless, they could be unmasked by drawing out the excess of myeloid cells with aleuronat. He, however, accepted the general concept that both types of centers were extravascular in the mammal.

Cunningham and Doan (73) showed that erythropoiesis could be completely unmasked in the rabbit by repeated intravenous injections of inactivated typhoid bacilli; these experiments were extended (Doan *et al.*, 23) by more elaborate methods for studying erythropoiesis. Since it was known that bleeding gives a hyperplastic marrow, while transfusions lower the rate of erythropoiesis, a series of matched donors and recipients were used for repeated transfusions, and in these two groups Doan and his co-workers studied the formation of the red cells in active and quiescent state after withdrawing the myeloid elements as before, namely, by injections of inactivated typhoid bacilli. In these experiments the swollen endothelial cells of the intersinusoidal capillaries were clearly demonstrable as the first step toward erythropoiesis, after a state of hypoplasia. This swelling of the endothelial cells has been seen before and was described by Maximow (21) in 1909, without, however, the same interpretation. The relation of the megaloblast, as the first step of erythrogenesis, was also shown to be as true for the mammal as for the bird. As erythropoiesis proceeds in the recovery from hypoplasia, erythroblasts develop from the megaloblasts and form the center of small groups, the megaloblasts always retaining their original position against the endothelial wall (fig. 36 in article by Doan *et al.*, 23).

In normal mammalian marrow megaloblasts are either absent or present in but small numbers, so that it is the early and late erythroblasts that form the edges of the groups of developing red cells with normoblasts in the center of the lumen of the capillaries, which dilate to accommodate the cells as they increase in numbers. Along the edges of such clumps, endothelial nuclei, sometimes swollen, can be made out, but as the process continues the endothelial cells are stretched until they become very thin. This concept of the normoblasts being in the

center of the clumps is the reverse of the description given by Bunting (24), who interpreted the erythrogenic clumps as having a border of normoblasts around clumps of immature red cells. In the early stages of regeneration, as well as in the unmasking of normal erythropoiesis, both erythroblasts and normoblasts are often found in single rows, filling the lumen of a capillary, and these single rows of developing red cells, outlining the regular pattern of a vascular network, are convincing evidence of their intravascular origin and development. In sections of active bone marrow, the myeloid cells often seem to be resting directly on the clumps of intravascular red cells, which is inevitable with the great crowding of the cells, and hence, to see clearly the intravascular position of the immature red cells, one must resort to methods for unmasking erythropoiesis by depleting marrow of its myeloid elements.

The concept that the immature red cells develop in erythrogenic capillaries gives a clear idea of how the inert red cells get into circulation. It is obvious that the red cell does not enter by its own motility. Under the prevailing theory that the inert red cells originate outside vessels, it has been thought that either the circulation of mammalian marrow is always open, or that as the red cells develop against the outer wall of the sinusoids, they bend and break the thin walls (Maximow, 21). It was then postulated that a growth force pushes the red cells through constant or temporary openings into the blood stream. If, however, the concept that the developing red cells are within capillaries, temporarily closed to the circulation, proves to be correct, then it is easy to see how these capillaries, when distended with normoblasts, might open up enough first to let the plasma in between the normoblasts and then to sweep these capillaries empty of their newly formed red cells. Such an opening of closed capillaries would be entirely analogous to the proved opening of collapsed capillaries elsewhere in the vascular system (Krogh 4; Hooker, 74, 75; Rich, 76; Richards, 77; Richards and Schmidt, 78; Bayne-Jones, 79). It has been shown by Key (80) that the immature red cells have a quality of stickiness in their surface membranes which disappears just as the normoblast becomes the red blood corpuscle. The exact stage of the loosening of the clumps of normoblasts by the plasma filtering in around them has been demonstrated by Doan *et al.* (23; see their fig. 35), who unmasked erythropoiesis in the rabbit and then injected the marrow with India ink. The sinusoids and their erythropoietic capillaries could then be seen with continuous and intact endothelial linings, because there were no myeloid cells along the outer

border; in these specimens the ink from the sinusoids could be seen as it had just entered the hematopoietic capillaries, and was found sticking to the normoblasts in their lumen. A similar experiment, but without the unmasking of the vessels, was made by Drinker *et al.* (22). They took a rabbit in which, after repeated bleedings, the marrow was in an active state and selected a time when the peripheral blood showed a cycle of delivery of both red and white cells into the circulation, and immediately made an injection with India ink. In this stage there were places of irregularly outlined carbon, apparently extravasation, which, however, were not general, but rather sharply localized, so that they described the ink as being held between columns of cells upon which it seemed to border. Lacking the depletion of the myeloid elements, the endothelial boundary enclosing the young red cells was not so obvious, but their figure (fig. 37 in Drinker *et al.*, 22) can be interpreted as the entrance of of ink into the intersinusoidal capillaries just opening up to the circulation, and is in striking contrast to their injections of hemorrhagic marrow after saponin (figs. 43 and 44).

The concept of the opening of erythrogenic capillaries at certain periods to admit the maturing red cells to the circulation gives a feasible explanation of normoblastic crises from a premature opening of such capillaries to the circulation. Under conditions of extreme need for the regeneration of red blood cells, megaloblasts can also be found lining the walls of patent sinuses.

After their studies of unmasked erythrogenesis in the rabbit, Doan *et al.* (23) also found that the same processes of regeneration from hypoplasia could be traced in occasional opportunities in human marrow, reporting a case of typhoid fever, one of starvation in the insane, and two of bacterial endocarditis.

The process of the origin of red cells from endothelium was then studied in the human by Peabody (81) in a series of cases of typhus fever and illustrated from one case in which the preservation of the tissue and the technique of preparing the serial sections were faultless. Peabody brought out that the pathologist, as a matter of fact, has relatively frequent opportunities to study erythropoiesis alone in human material, under conditions when the marrow of the long bones, normally aplastic, is beginning to take on hematopoietic function. This occurs not only in recovery from anemia, but also with most infections and intoxications (Naegeli, 82). Muller (83) was able to produce an hypoplastic anemia in rabbits by collargol and by regulating the doses and varying the time of killing the animals after the last dose, she obtained material both in

the aplastic state and in the phases of regeneration of both red and white cells. She also showed (84) that with injections of India ink there was such a stimulus to the formation of red cells that the open sinuses were lined with nucleated red cells. To this material, Doan and Sabin (85) have added the study of the recovery from anemia of a series of tubercular rabbits. They found that after massive doses of bovine bacilli, the marrow became simplified, that is, relatively aplastic for blood formation, on account of an extensive encroachment with tubercles, but that the marrow tended to recover in every case in which the animal survived the acute phase of the disease, regardless of the extension of the tubercular processes elsewhere. The platelets started to regenerate first, then the red cells, followed by the granulocytes.

In recent experiments with amphibian marrow, Jordan and Baker (26) have found the same opportunity to study erythrogenesis alone, since the stimulus in the frog's bone marrow after splenectomy is limited to the production of red cells. It is also interesting to note that Koolman (86) reports that the removal of the bone marrow is a stimulus to regeneration and that in the stage of recovery after removing the radial bone marrow in dogs, the red cells began to rise in the circulating blood, while the white cells were still falling.

In all this material, the avian, the experimental mammalian, and the human, it is evident that the initiation toward regeneration from aplasia begins with erythropoiesis, rather than with an equal start in the production of red and white cells. Thus, beginning with the earliest stages of regeneration from a hypoplastic state, it is possible to study first erythropoiesis and later granulopoiesis in separate foci. Where erythrogenesis is thus simplified, it can be seen to be intravascular in type, with a swelling of endothelium as the preliminary phase. There is one dissenting opinion on the interpretation of such experimental material. Jordan and Baker studying such simplified erythropoiesis in amphibian forms, interpret the thin cellular borders which they also found around developing red cells as made by flattened reticular cells having no relation to endothelium. With this exception, the more recent workers with simplified material (Doan, Cunningham, Sabin, Peabody, Muller) agree that the evidence is for the origin of the red cells from endothelium and the intravascular maturation of red cells in mammalian material.

It thus appears that the observation of the presence of erythrogenic and leucogenic centers in marrow, starting with Muir, Selling, and Bunting, finds its interpretation in the concept that the red cells develop in erythrogenic capillaries closed to the circulation; that the complete

separation of the two processes, one within vessels closed to the circulation, and hence in places of low oxygen tension, and the other between the dilated sinuses and thus provided with an abundant and sluggish flow, can be demonstrated both in the recovery from aplasia and by the unmasking of normal erythropoiesis by methods which remove the excess of myeloid elements. When one has become familiar with such material, the same structural relationships can be seen in normal and even in hyperplastic marrow, in spite of the crowding of myeloid elements around the erythrogenic capillaries. It is important to note that the red cell is the first of the blood cells to form in the embryo and throughout life the regeneration of red cells precedes regeneration of the white cells from an aplastic state of bone marrow.

IV. Formation of white blood cells in marrow. *Maturation.* It is known that granulocytes, that is, neutrophilic, basophilic, and eosinophilic leucocytes, arise outside blood vessels and pass into them by their own motility. The cell from which they come is a matter on which there is difference of opinion. According to one concept of the origin of blood cells, there is a common stem cell for both red and white strains, which is a basophilic, polyvalent form. This hypothetical cell has had many names (the mesamoeboid cell, the lymphocyte, the lymphoidocyte, the hematoblast, the hematocytoblast, etc.), resulting in a condition in which the nomenclature has become more complicated than are the facts.

While a science is in a descriptive phase, complex terminologies arise and can be carried, but as a subject becomes experimental, it is essential to have criteria that can be readily compared. To this end the factors of maturation of granulocytes have been expressed in a diagram. On chart 1 maturation is represented as a series of varying factors, constantly changing; in some levels, the factors may increase together, in others some may increase while other decrease. The cells are shown in three levels, the original non-granular stages, the granular marrow cells or myelocytes, and the leucocytes, the three levels being separated by two critical points, shown as solid vertical lines. The first marks the point of the beginning of granulations and the second that at which the last cell division of the myelocyte has taken place, with the reduction to a cell of common size and state of maturity, the definitive leucocyte.

Level I. The cells of level I can best be studied in simplified marrow. In completely aplastic adult bone marrow, such as that of the pigeon after a short fast (3), from which active marrow is readily and quickly regenerated, only three types of cells can be seen: fat, endothelium, and

reticulum. Of these three, the fat is but accessory to blood formation. Evidence has just been given that the red cells regenerate from the endothelium. The white cells come from the reticulum.

The reticular cells are easily found both in fresh and in fixed aplastic marrow, since they are the only type in the interstices between the fat cells. They are few in number and are scattered along the reticular framework. Their lack of differentiation gives few characteristics by which to describe them. The cytoplasm is faintly basophilic and shows

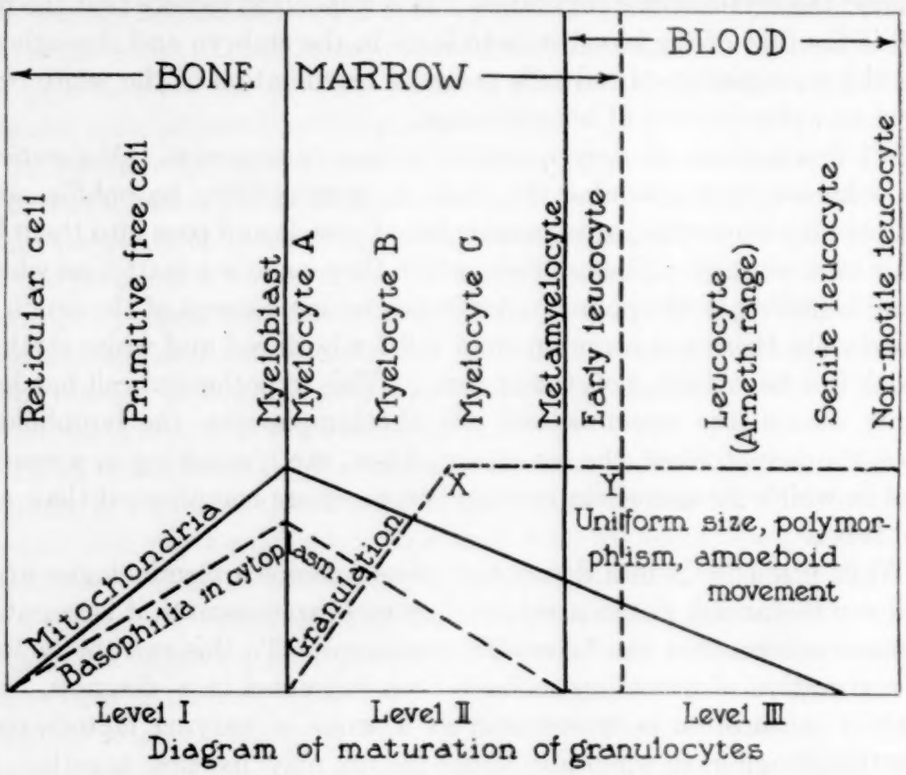

Chart 1

no granulation, not even mitochondria; if mitochondrial substance is present, it is too finely divided to be seen; the nuclei have so little chromatin as to make them scarcely more basophilic than the cytoplasm. These cells, the so-called reticular type, are probably as close to primitive embryonic mesenchyme as occurs in the adult animal. In this sense, the term reticular cell is used for a type less differentiated than the fibroblast (Mallory, 87), but as a form which may also differentiate into a fibroblast. Their characteristics and their relation to the origin of the white blood

cells were described by Cunningham *et al.* (88) and illustrated as individual cells in their figures 2 to 5, plate I. They are well shown by Orsós (89) in his figures 1 and 2, in their relation to the reticular fibrils in the interstices between fat cells in sections of human marrow from a case of aleucemic leucemia. These cells are not an hypothetical form; they can always be found in bone marrow, lymph glands, and spleen. They have been described by Lang (90) as cells of embryonic type along blood vessels, in contrast to the adventitial cells of Marchand, the so-called phagocytic clasmatocytes. Both the reticular cells and clasmatocytes can be found along the blood vessels in the connective tissues, and both have been illustrated by Kubie (91) along the vessels of the brain. He found the reticular cell as a normal type in especially large numbers along the vessels of the nervous system and used the term lymphoblast, because in this location they are the forerunners of the lymphocytes in the perivascular reactions of the nervous system. The above observations demonstrate and show how to find the actual undifferentiated reticular forms which are the type long postulated by hematologists as the forerunners of the blood cells.

In regenerating marrow, this primitive reticular cell, with repeated divisions, gives rise to a primitive free cell. In this process a few mitochondria are elaborated in the cytoplasm and a basophilia is gradually built up. The stage of the primitive free cell is shown by Cunningham *et al.* (88) in their figures 6 to 10, and by Orsós (89) in his figures 3 and 4. Finally, as the basophilia of the cytoplasm and the numbers of the mitochondria reach a maximum, the cell becomes the myeloblast (chart 1).

Naegeli (92), to whom is due the concept of the myeloblast, does not so limit the term to the stage of full maturity in this level; but the convenience of such a limitation of the term lies in the fact that this is the cell of myeloblastic leucemia. Since this period is one of growth and division, as well as one of maturation in regard to mitochondria and basophilia, the cells are to be found in varying sizes; but in normal bone marrow, it is the primitive free cell of small size, similar to the small lymphocyte, but differing from it in having fewer mitochondria and a less differentiated nucleus, which is to be found in the largest numbers. It is this small cell which has been confused with the mature small lymphocyte, which has been so great a stumbling block in the understanding of the mechanism of bone marrow. The reason for the difficulty is clear, for the morphological points of distinction between the primitive free cell and the small lymphocyte are not striking; it is now certain that lymphocytes may arise in bone marrow both under normal and under

pathological conditions, but the usual cellular output of the marrow is limited to red cells and the three strains of the granulocytic leucocytes, so that the primitive free cell of bone marrow normally produces granulocytes in that location.

Level II. The classification of the myelocytes, level II, by the number of the granules was introduced by Sabin, Austrian, Cunningham and Doan (93) from a study of leucemic blood. Myelocyte type A is derived from the myeloblast, of maximum basophilia and mitochondria, by the addition of the first small clump of granules; type B represents the stages during which granulations are increasing in number; while type C is the stage when they have reached their maximum. These three stages are readily discriminated in any technique, but the earliest granulations, as soon as they are large enough to be visible, are more readily seen in fresh, unstained marrow or after supravital staining than in fixed films, where they may be obscured by the marked basophilia of the cytoplasm. However, with careful technique, they are visible in methylene-blue-azur, as shown in Pappenheim's *Atlas of Myeloid Cells* (94). The conventional classification of level II is promyelocyte and myelocyte, based on the changing chemistry of the granules; the relative value of the two classifications will be discussed later in relation to their use in the study of marrow as an organ.

During the stages of level II, the basophilia and mitochondria built up in level I gradually disappear as the granules increase in number. The vertical line at the end of level II is based on one point, namely, on the last cell division. Around the second critical line in chart 1, at the end of level II, two factors vary, the cessation of the basophilia and the beginning of ameboid activity; both are shown at the line merely for the sake of simplicity. In general, the basophilia does disappear at the end of level II, but a slight basophilia may be carried over to the early leucocytes normally and, in conditions of leucocytosis, where young leucocytes are drawn quickly into the blood stream, as in pneumonia, the young leucocytes of the blood are often still basophilic.

Concerning the onset of the ameboid movements, the term metamyelocyte is convenient; the myelocyte C shows no movement of the granules in the fresh state; the cytoplasm is probably too much of a gel to allow of the streaming of the granulations, which makes possible typical ameboid locomotion. Myelocyte C may show slight locomotion, but with no such speed as that which characterizes ameboid movements. The stage of the metamyelocyte from human blood was shown by the writer (Sabin, 95), under the mistaken label of "large mononuclear"

in supravital reaction. It is frequently found in small percentage in the circulating blood of people practically normal, as confirmed by Shaw (96) and Stetson (97). Nevertheless, it is a cell of the marrow and its presence in the blood probably indicates some absorption of bacterial products. The very beginning of ameboid activity is often seen in the metamyelocyte as a slight local shifting of the granules, as the cytoplasm starts to become more fluid; this property, together with a slight indenting of the nucleus, discriminates the type from myelocyte C, but the larger size separates the cell from the leucocyte and possibly it is the stage just before the final division. In the leucopenia of typhoid fever, the myelocyte B may skip the stage of myelocyte C, and still having marked basophilia, but deficient granules, may begin to show streaming of granules and appear in the circulating blood. Thus there are normally slight variations in the point of the disappearance of the basophilia and the onset of ameboid movement around the line at the end of level II, which may become quite wide variations under abnormal conditions.

As is shown in chart 1, the mitochondria gradually diminish in number; at the same time they also become smaller, and finally disappear entirely at the stage of senility. Throughout level II there are both growth and cell division, so that there is variation in the size of the cells with a tendency for myelocytes C and metamyelocytes to be large forms.

Level III. The third level, marking the end of maturation, is characterized by cells of approximately uniform size and content of the specific granulation. These factors mean functional maturity. The uniformity of size is due to the cessation of division which precedes the stage of polymorphism; the nuclei fragment and the cell frequently drops off bits of cytoplasm, but division no longer takes place. As is well known, ameboid movements are a constant phenomenon of the leucocytes, whenever they have a surface to move upon. The leucocytes become more mature in the circulating blood according to the Arneth pattern (98), involving the nuclear changes, and finally pass into the non-motile phase (Sabin), in which the granules no longer stain supravitally in neutral red; the cells in this stage are the fragile forms with easily ruptured membranes in fixed films. Cells in the non-motile phase appear in the blood in showers (Sabin, Cunningham, Doan and Kindwall, 99), indicating that leucocytes in part die in the blood stream. In part they pass out into the tissues, a point which will be discussed later.

Chart 1 is based on cytoplasmic criteria. There are also nuclear criteria. In general, immature phases of both the red and the white strains of cells have less chromatin and possess true nucleoli, plasmasomes, con-

sisting of pyronin (Schridde, 100) rather than condensations of chromatin. With this exception, differences in nuclei are more sharply differential for discriminating the erythroid from the myeloid strains (Türk, 101), and for separating myeloblasts from lymphoblasts (Schridde, 100), than for tracing the stages of development within a single strain.

Relation of granulocytes to the vascular pattern. The process of the regeneration of the white cells in aplastic marrow is different in place, in time, and in method from that of erythrogenesis (Doan *et al.*, 23). In the earliest stage of regeneration of red cells, the entire gelatinous marrow is seen to have a low blood supply with but few patent sinuses and many collapsed ones. Along the border of such a marrow there is a narrow zone, where the blood vessels of the marrow anastomose with those of the shaft of the bone. As granulopoiesis begins, every vessel of this narrow border is to be found patent to the circulation, that is to say, there are no collapsed intersinusoidal capillaries. No other area of the marrow has so constantly a maximum blood supply, and it is in this narrow strip that the onset of regeneration of white blood cells is to be found. The contrast between the central, relatively avascular, erythropoietic zones and the marginal, vascular, granulopoietic zones is well shown by Doan *et al.* (23) on their plate II. In this area can be followed the stages of maturation, through the three phases or levels just described —the primitive, the myelocytic, and the leucocytic.

In their relation to blood vessels, it will be seen that in granulopoiesis the conditions are the exact reverse of erythrogenesis. With the red cells the most immature forms are against the endothelium on the inside of the vessel; with the leucocytes, on the other hand, it is the most mature forms, myelocytes C, the metamyelocytes, and the leucocytes, that border vessels, and the metamyelocyte and still more the leucocyte that enter the lumen.

In the beginning of regeneration from the aplastic state, the difference in time for the regeneration of red and white cells, and the difference in place allow for a separation of the two processes; the initiation of the formation of red cells is in the center of the marrow in zones of low circulation and always precedes the start of granulopoiesis. Shortly afterward, granulopoiesis begins along the border zone in the area most richly supplied with blood.

It is only for a brief period that there is this complete separation in the zones for the formation of red and white cells—the more central, less vascular zone of red cell formation and the peripheral, vascular zone for the formation of the white cells. Soon the two processes become

more diffusely represented throughout the marrow, but always the fundamental relationship is maintained that the white cells develop around the patent sinuses and move toward their borders as they become more mature, while the red cells, on the other hand, develop in the areas of the closed sinuses, which, however, are connected with the patent sinuses through potential openings. In normal marrow the vascular and the avascular areas are thus close together instead of being peripheral and central in the marrow as a whole, as in the early stages. A peripheral, vascular, and thus granulopoietic area is maintained in the marrow of the adult rabbit, and hence remains the zone in which the status of leucogenesis can be studied in its simplest form.

The question of the myeloblast. Around the cells of level I, as shown in table 1, have waged the discussions concerning cell potencies, so be-

TABLE 1

LEVEL	BONE MARROW	LYMPH GLANDS	SPLEEN AND GENERAL CONNECTIVE TISSUES
I	Reticular cell Primitive free cell Myeloblast	Reticular cell Primitive free cell Lymphoblast	Reticular cell Primitive free cell Monoblast
II	Myelocyte A Myelocyte B Myelocyte C Metamyelocyte	Lymphocyte	Early monocyte Mature monocyte
III	Leucocyte		

wildering that Schridde (100) quotes Türk (101) as "saying resignedly" that "each one must have his own stem cell." Concerning the primitive reticular cell, primitive mesenchyme, whether in bone marrow or elsewhere, all observers might well meet on common ground as far as its power to give rise to the three strains of white blood cells—granulocytes, lymphocytes, and monocytes—is concerned, inasmuch as these cells are all mesenchymal in type. Beyond this point, the discussions center around the problem of the myeloblast, which has recently been reviewed by Downey (102), with a full bibliography. The cell later known as myeloblast was first described by H. F. Müller (103) from leucemic blood. The concept of the myeloblast announced by Naegeli (92) in 1900 was, as Schilling has said, the beginning of light for this vexed question. The problem, if still at issue, is exactly where to place the power

of trivalency; does it still exist in the primitive free cell, and have myeloblast, lymphoblast, and monoblast become determined toward the mature forms to which they correspond? For the discrimination of myeloblast and lymphoblast, there are three criteria: first, differences between the nuclei; second, the patterns of mitochondria, and third, the association with neighboring cells in the process of maturation (chart 1). Schridde (100) showed that the nuclei of the lymphoblasts have the denser membranes and the coarser chromatin network. For the cytoplasm there are the degree of basophilia and the number, size, and distribution of mitochondria. Differences in shape of mitochondria between myeloblasts and lymphoblasts have been described by Naegeli (92) and Schridde (100) in fixed films; differences in arrangement by Sabin *et al.* (93), Cunningham *et al.* (88), and by Simpson and Deming (104), from supravital studies with Janus green. How constant these patterns will prove and what their range of variation is must await further study.

In the study of leucemic blood, the actual diagnosis of myeloblastic leucemia is to be made, not because the morphological criteria, nuclear and cytoplasmic, just described are so clear and convincing as to admit of no differences in opinion, but rather because myeloblasts do not occur entirely alone, but rather in association with some young myelocytes, those which show the onset of the next level, our type A, or myeloblasts with the so-called azurophilic progranulations. This was shown by Sabin *et al.* (93) in a case of myeloblastic leucemia. It has been confirmed with supravital studies by Simpson and Deming (104), who find in fact that in the blood of some cases of myeloblastic leucemia most of the cells may actually be the form of our myelocyte type A. It was also shown by Sabin *et al.* (93), confirmed by Richter (105), that it is the myelocyte type A that gives the meager but positive oxydase reaction, in contrast to the entirely negative reaction of myeloblasts. For the monoblast also the practical discrimination of the type is to be made in tissues through the actual association of the more differentiated types along with the immature. The value of the morphological criteria, nuclear and cytoplasmic, for discriminating immature blood cells is clear; these criteria, taken together with the fact that no one type in the constantly shifting stages of maturation, indicated on chart 1, occurs completely isolated from the adjacent stages, give data which are usually adequate for the diagnosis of the type of leucemia. The more theoretical question, perhaps the physiological problem, of the analysis of how far myeloblast, lymphoblast, and monoblast are already deter-

mined toward their mature strains must await the experimental evidence which will come as the nature of the chemical substances which control maturation is discovered.

Lymphocytes in bone marrow. With the simplification of our concepts of the structure of bone marrow, which began with the introduction of Naegeli's concept of the myeloblast, and with the realization that the primitive cells of the bone marrow are not identical with mature lymphocytes, the question of the occurrence of lymphocytes, not as scattered cells, but as developing locally in characteristic lymph follicles, becomes significant in relation to the question as to whether the primitive cells of the bone marrow are trivalent.

In 1902, Dominici (106) described rudimentary lymphoid follicles around the arteries of the marrow of a rabbit 15 to 20 days old; and since then the observation has been repeatedly made for human bone marrow. First, Longcope (107) reported finding follicles in human bone marrow in cases of typhoid fever; Hedinger (108) found germ centers in the bone marrow of a case of status lymphaticus; and Oehme (109) described typical lymphoid follicles with margins of mature lymphocytes and centers of pale cells, lymphoblasts without the Schridde-Altmann granules (mitochondria), in a series of cases of pneumonia, rickets, and diphtheria in children. Askanazy (110) found follicles in 43 out of 126 cases, and viewed their occurrence not as a pathological, but rather as an occasional normal phenomenon. He found them not only in areas of the formation of red cells, but also in zones of active granulopoiesis. This work was extended by Fischer (111), who studied 61 cases ranging in age from 6 months to 84 years, and found follicles in 62 per cent. She gives many photographs both of single and of multiple follicles. The occurrence of lymph follicles has thus been amply confirmed (see also Aschenheim and Benjamin (112) and Weidenreich (113)), so that the possibility of the development of them in bone marrow may be considered as established, which brings evidence to bear toward the theory that the primitive cells of the marrow are trivalent.

In these studies it is striking how many of the observations have been made upon children, and especially in cases of rickets, but in the surveys of Fischer the proportion of children with follicles was not greater than that of adults. The subject of the differences in the marrow of children and adults will be taken up later.

Monocytes in bone marrow. The monocyte does not occur in normal marrow in any large numbers, but monocytes can be stimulated to arise there. Doan and Sabin (85) have demonstrated this by massive injec-

tions of bovine tubercle bacilli in rabbits; the bone marrow becomes extensively involved with tubercles in the first three weeks. They made supravital studies of the marrow every day after the injection of tubercle bacilli; on the eighth day they found small clusters of the earliest monocyte that can be recognized, one with a single row of the characteristic neutral red bodies around the centrosphere, corresponding to myelocyte A, and these young monocytes were intermingled with monoblasts and mature monocytes; at the same time the fat was beginning to disappear from the marrow. In succeeding days, the monocytes increased in numbers and in maturity and from them came typical epithelioid cells making tubercles.

To the fact that both lymphocytes and monocytes may arise in bone marrow it must be added that granulocytes may also arise in lymph glands (to be discussed later under "ectopic blood formation"). However, these facts do not alter the concept, which goes back to the work of Ehrlich, that normal adult bone marrow produces the granulocytes of the blood and the lymph glands the lymphocytes.

V. Bone marrow as an organ. It might be thought that the study of bone marrow as an organ should have begun with the discovery that blood cells arise there and with the pioneer work of Ehrlich; but following these early discoveries, attention was necessarily concentrated on the processes of maturation of red and white cells. Our knowledge of the evolution of blood cells has been gained more from the study of embryological stages before bone marrow functions, and far more from the study of peripheral blood, both in infections and in anemia and in leucemia, than from the study of normal bone marrow. As a result, the idea of studying bone marrow as an organ is relatively modern, and, inasmuch as the study of complex pathological marrow preceded a clear concept of its normal mechanism, a false idea has gained ground that marrow has a bewilderingly complex structure. Perhaps it is even simpler than subcutaneous tissue, for certainly fewer strains of cells are involved.

This modern phase of hematology, the period during which differential counts of the cells of the marrow have formed the basis for analysing the functional state of the organ, could not begin until certain fundamental points had been established. Three of these points have already been discussed: the clarification of the processes of maturation until there was a common basis of classification; the analysis of the nature of the vascular pattern of marrow, showing that collapsed capillaries favor erythrogenesis and dilated sinuses granulogenesis; the theory of the myeloblast as demonstrating that the output of the marrow in white

cells is normally limited to granulocytes, with lymphocytes and monocytes not involved. Beside these three factors, there is a fourth one of great importance, namely, an exact measure for the output of marrow. It has been found that there are various physiological outflowings of white cells from the blood into the tissues. Stockard and Papanicolaou (114) showed a marked migration of leucocytes into the vagina in guinea pigs at a certain stage of estrus; Isaacs and Danielian (115) have demonstrated an elimination of leucocytes into the saliva, which is probably indicative of a phenomenon in the entire digestive tract; Bunting and Huston (116) demonstrated a similar migration of lymphocytes into the intestines. Beside these reactions, Barcroft *et al.* (58) found temporary storage of red cells in the spleen; and Goldscheider and Jacobs (117), discussed later, introduced the subject of redistribution phenomena of white cells in pathological reactions. All these observations make it clear that total and differential counts, giving the data for the number of cells in a cubic millimeter of blood, are alone insufficient to demonstrate an increased or decreased output from the marrow. An actual increase in immature forms must be known, the adequate criteria for which are the Arneth (98) "shift to the left" in the nuclear pattern for leucocytes and the reticulated count for the red cells.

The earlier of the modern studies of bone marrow involving differential counts to detect pathological changes were made by Rubinstein (118) in 1901, Price Jones (119), Wolownik (120), Bätge (121), Aschenheim and Benjamin (112) and Lossen (122). Rubinstein detected the shift to the less mature myelocytes in the marrow which is so constant a reaction under abnormal conditions. Lossen made counts of the total number of cells per cubic millimeter in children's marrow, finding that they ranged from 270,000 to 1,568,000 and that their number seemed, in a general way, to be dependent on the state of nutrition. The subject is one of extreme difficulty for numerical estimation, because of the uneven distribution of the zones of marrow between fat cells, but a general idea of the amount of marrow in terms of increased or decreased fat cells is of great importance; if Lossen's point of a decrease in the number of cells in the marrow of children under malnutrition be confirmed, it will have practical bearing. In this connection should be studied the results of Suzuki (123) on the blood-forming organs during inanition in guinea pigs. He found a lymphopenia corresponding to an atrophy of the lymphoid tissue generally correlated with a leucocytosis of the pseudo-eosinophiles, due to a stimulus both of the marrow and to a granulopoiesis in lymph glands.

The more comprehensive recent studies on the subject of marrow have been made by Naegeli (82), Müller (51), Arneth (124), and especially by Schilling (125) and his students Bantz (126) and Yamamoto (127). Naegeli's studies led him to classify pathological changes of marrow into four types: erythroblastic, myelocytic, myeloblastic, and lymphatic. Schilling's classification involves five general types: 1, ripe neutrophilic; 2, unripe neutrophilic; 3, ripe promyelocytic; 4, unripe promyelocytic, and 5, myeloblastic, to which are to be added the megaloblastic form of pernicious anemia, the lymphoid type of lymphatic leucemia, and the aplastic state, as special forms.

The studies of our group have been with normal bone marrow and with experimental studies that may be considered relatively normal (Cunningham and Doan, 73; Doan *et al.* 23; Doan and Zerfas, 128; Doan and Sabin, 85; Sabin and Doan, 129). The subject of bone marrow as an organ means the consideration of the entire marrow as a unit. It is an organ of no inconsiderable size, having been shown by Wetzel (130) to have a volume, in the adult human, of 1419 cu. cm., which is thirteen times that of the spleen, and almost equal to that of the liver.

Considering all of the marrow as a unit that is functioning in the formation of blood at any one time, that is, the entire marrow of rabbit or guinea pig or all the red in man, the whole structure will be found in a relatively uniform state under normal conditions. Since the marrow is carrying on two different processes, this statement means that the proportion of erythrogenesis to granulogenesis is relatively constant, as well as the proportions of the different levels of maturation within the two groups. This condition is not true when any deviation from the normal has brought about an extension of red marrow into the yellow, since there, first erythrogenesis, and later granulopoiesis will be found at earlier levels.

Comparative studies of blood and bone marrow for a group of five normal and two slightly abnormal rabbits were shown in three tables given by Sabin and Doan (129). A preliminary count of the blood was taken immediately before killing the animal, which was done by an injection of air into the veins. Normal marrow can only be analyzed with specific reference to the state of the blood of each animal; if other factors, peripheral redistribution and ectopic bone marrow, are ruled out, then the correspondence between blood and bone marrow will be found to be relatively exact.

For the surveys of the marrow, supravital preparations were made from the center of the shaft and from the epiphysis of each of the long

bones from either side, and one preparation from a rib, making nine slides in all. From these preparations, from four to seven thousand cells were counted by the two observers. There was no constant difference between erythropoiesis and granulopoiesis for shaft, epiphysis and rib, but because there are two processes occurring in different foci, it was found advisable to count about five thousand cells for comparable results.

In making the preparations, a small bit of the marrow was taken, avoiding the peripheral area of predominantly leucocytic function, and the areas between the fat cells, as well as some of the free cells along the border, were counted. For the zones between the fat cells, it was necessary to select areas that were relatively small, so that each cell could be identified; in avoiding the denser areas, it is probable that the number of the primitive forms of the white strain have been underestimated. The difficulties of the counting, due to the large number of cells and to the structural variation from two types of centers, make it hard to get surveys that are representative. The actual percentages connot be regarded as even as close an approximation to the actual as blood counts, which themselves are likewise only approximations; nevertheless, the correspondence of the findings in the different animals to their own peripheral blood indicates that the results, if expressed in general terms, show a normal level for bone marrow as an organ.

The concept of the formation of white and red cells, given in the preceding pages, simplified such an experiment. This is true because all the stages of maturation of the red cells, the megaloblasts, erythroblasts, and normoblasts, as well as the preliminary endothelium, are readily discriminated in either fixed or supravital technique. For the fixed technique it is, of course, essential to be able to fix marrow before autolysis has taken place, and to use a fixing reagent without acid, for example, Helly's solution, in which neutral formalin is substituted for the acetic acid of the Zenker solution. For the white cells the classification given above makes the counts feasible, for the cells are considered in three groups, the agranular forms, the granular marrow cells, and leucocytes, with recognition of the range of variation in each group. From the data two points are to be analyzed, first, the normal proportion of erythroid to myeloid activity, and second, the proportions of the different stages within the two groups.

Ratio of erythropoiesis to granulopoiesis. For all seven animals the total red counts of the peripheral blood were normal (5,500,000 for the rabbit), but in one, 19B, the fat was disappearing, indicating an exten-

sion of hematopoietic tissue, which involved an increased percentage (37) of red cells. With this exception, the ratio was about 70 per cent granulocytes to 25 per cent erythroid cells, with all other cells 5 per cent (table 2). Thus, for the normal rabbit, there are approximately three times as many myeloid cells as erythroid in marrow, as contrasted with a thousand times as many red cells as white in the peripheral blood. This reverse in the ratio must be correlated with the greater length of survival of the red cells in the blood stream. Schilling (125) with Banzler had an opportunity during the war to study the marrow of ten healthy men, in whom counts of the blood had been made before death, and they found 44 per cent neutrophils plus eosinophiles (types not given) to 36 per cent normoblasts in the marrow. Doan and Zerfas (128) give the following myeloid-erythroid ratios for three accident cases: 80:5, 80:4, and 71:13;

TABLE 2

RABBIT NUMBER	PERIPHERAL BLOOD			BONE MARROW			NUMBER OF CELLS COUNTED
	White blood cells	Myeloid cells	Red blood cells	Total myeloid	Total erythroid	All other cells	
		total number		*per cent*	*per cent*	*per cent*	
1B	15,900	42% (6678)	6,360,000	72	26	2	4800
2B	12,600	40% (5040)	6,130,000	71	27	2	4200
3B	14,900	45% (6705)	5,800,000	71	19	10	7500
6B	11,900	48% (5712)	6,230,000	71	21	8	7300
7B	8,900	46% (4094)	5,230,000	68	28	4	6500
13B	12,200	83% (10,120)	5,610,000	63	27	10	4800
19B	14,800	71 (10,508)	6,050,000	59	37	4	5900

their myeloid cells include leucocytes, myelocytes, and myeloblasts. To establish the limits of variation of this ratio under normal conditions would be important.

Proportion of the different levels in granulocytes. The data concerning the myeloid elements alone are shown in table 3. Five of the rabbits had entirely normal blood, with total myeloid cells averaging 44 per cent, of which 36 per cent were pseudoeosinophiles (range 33 to 39 per cent), 7 per cent were basophiles (range 4 to 11 per cent), and 1 per cent were eosinophiles (range 0 to 2 per cent). The other two animals were clinically normal, but were chosen for the study because of the increased percentages of granulocytes; R 13 B had 71 per cent pseudoeosinophiles, 10 per cent basophiles, and 2 per cent eosinophiles, making total myeloid cells 83 per cent; while for R 19 B the corresponding figures

were 68, 2, and 1, with a total of 71 per cent. For the five normal rabbits, myelocytes C averaged 85 per cent, with myelocytes A and B, and myeloblasts in minimal numbers. Making due allowance for the limits of error in marrow counts, it is clear that normal marrow shows striking predominance of the myeloid cells near maturity.

The condition of the marrow in the two animals in which the blood had indicated hyperactivity of the marrow in pseudoeosinophiles was striking. For R 19 B, the proportion of myelocytes C (54.43 per cent) had been diminished both by an increase in leucocytes up to 19.99 per cent, meaning the greater delivery into the blood, and by an increase in myelocytes B up to 19.29 per cent from the normal of 4.36 per cent.

TABLE 3

Percentages of types of cells within the myeloid group in bone marrow

RABBIT NUMBER	POLYMORPHONUCLEARS	NEUTROPHILIC MYELOCYTES			MYELOCYTES		MYELOBLASTS	MYELOID CELLS COUNTED
		C	B	A	Eosinophilic	Basophilic		
1B	3.15	92.10	1.84	0.11	2.06	0.74	0	3,484
2B	7.34	79.38	8.35	0.43	2.37	1.57	0.56	2,988
3B	3.96	87.33	3.46	0.58	2.10	0.40	2.17	5,277
6B	1.93	90.87	3.16	0.36	1.22	0.84	1.62	5,215
7B	7.13	78.33	5.09	0.56	4.58	2.98	1.33	4,358
Average for five normal rabbits..	4.66	85.72	4.36	0.41	2.45	1.29	1.11	21,322
19B	19.99	54.43	19.29	0.76	2.36	2.95	0.22	3,514
13B	8.78	32.27	48.59	2.87	3.60	2.97	0.92	3,027

These cells were rather close to the C type, having more than half the full quota of granules. In the other rabbit, 13 B, the shift to immature forms was even more marked. Myelocytes C were 32.27 per cent, type B 48.59 per cent, and type A 2.87 per cent. In this animal myelocytes B were less mature, having on the average less than half the full quota of granules. These two animals represent the condition of bone marrow corresponding to leucocytosis; it consists in an increased percentage of the early leucocytes (chart 1), together with an increased maturation of myelocytes from the earlier levels.

Reciprocal relationship of myelocytes C and early leucocytes. A point of significance in the study of the function of marrow follows from the predominance of the type C myelocyte, inasmuch as the delivery of

cells from the marrow is made from this level. It was shown by Sabin *et al.* (99), in a study of human blood, that leucocytes enter the blood stream rhythmically with small hourly accessions and a larger daily rise in the afternoon. It was then demonstrated by Shaw (96), under the interesting title of "The diurnal tides of the leucocytes" that the afternoon rise is but a part of a diurnal rhythm, with a second rise about midnight. This rhythmic pattern in the delivery of cells was shown by Doan and Zerfas (128) to persist in cases with leucopenia or leucocytosis, the fluctuations being particularly striking during leucocytosis. They followed the Arneth pattern in seven cases, and demonstrated that these rhythms hourly and diurnal are actually due to an increased delivery of cells from the marrow.

The mechanism of the rhythmic delivery of cells from the marrow is in the reciprocal relationship between myelocytes C and early leucocytes. On chart 1, the legend *X-Y* on the line of granulations covers the group of cells which take part in this reciprocal relationship. These cells include myelocytes C and metamyelocytes on the left of the line, and the early leucocytes which Schilling (125), (131) has described under two names, *Jugendliche* and *Stabkern*, on the right. The classifying of the gradual changes of maturation (see chart 1) into fixed groups is just as difficult in the actual differential counting of cells as in the agreeing on a common nomenclature, and all figures from bone marrow must be studied with these difficulties in mind. In our counts the cells recorded as early leucocytes all showed active motility and hence are to be compared with Schilling's type *Stabkern* plus the few mature leucocytes from the circulating blood. The counts in table 3 show a range of from 3 to 7 per cent for early leucocytes for the first five animals, 8.78 per cent for rabbit 13 B, and 19.99 per cent in rabbit 19 B. Doan and Zerfas (128) give the following ratio of leucocytes to myelocytes in normal human bone marrow: 55:18, 10:55, and 13:54. The reciprocal relationship of myelocytes to early leucocytes is also shown by Schilling and Banzler for the ten normal human cases already mentioned. Their figures vary between the limits of myelocyte-leucocyte ratio of 34:56 and 47:44. Normal marrow has not been studied with reference to the variations in this ratio for a day, but it is probable that if marrow cells were taken by puncture in the early morning and just before the afternoon rise, the changes in the reciprocal relationship would be as clearly marked as the changes in peripheral blood, provided that enough cells were counted to offset the local nature of the phenomenon.

The approximate hourly delivery of cells to the blood suggests that

the time for the maturation from myelocyte C to leucocyte might be expressed in units of hours or minutes. Doan, Zerfas, Warren and Ames (132) have demonstrated that the pouring out of new cells from the marrow occurs in from forty-five minutes to two and a half hours after sodium nucleinate, adenin and guanin nucleotides. It would be interesting to know how long the supply of myelocytes C might last, provided there was no immediate replacement from immature levels. The records of Doan and Sabin (85) of the study of the blood in rabbits with massive, intravenous doses of bovine tubercle bacilli give data that are suggestive in this connection. In the development of the leucopenia and anemia it took on an average eleven days for the marrow to reach the lowest level of red and white cells. In every case in which the animal survived the acute reaction, there was a disappearance of the tuberculosis from the marrow by lysis of the epithelioid cells; the onset of recovery in marrow function was first in platelets, then in the red cells, and it took about fifteen days for the red cells but only about eleven for the leucocytes to reach their original level, after which both passed above the original level, corresponding to a hyperplasia of the marrow before the final return to normal equilibrium. Thus, the time for the maturation of myelocytes A through to the myelocyte C may possibly by expressed in units of days.

In the counts of the marrow cells of the rabbit of Sabin and Doan (129), it is interesting to note that the eosinophilic myelocytes predominated over the basophilic, 2.6 to 1.8, while the reverse ratio was true for the blood, with one eosinophile to seven basophiles.

In making the correlation between the differential counts of Sabin and Doan (129) with other counts in the literature, it is necessary to relate the conventional nomenclature of the cells of level II, promyelocytes and myelocytes, heretofore used, with classification of myelocytes by the number of granules into types A, B and C. Pappenheim's term "*promyelocyte*" means cell with unripe granules. When neutrophilic granules (pseudoeosinophilic in the rabbit) first become visible, they are tiny and are quite acid or basophilic. They are spoken of as azur-progranulations. The changing chemistry of these granules, as they ripen, makes a difficult and uncertain basis for classification. Considering the matter first in supravital technique: neutral red is a chemical indicator with a range in pH from 6.8 to 8.0. The basophilic granulations are the most acid of all, and are a scarlet red; the earliest neutrophilic granules are red, but never quite as far to the acid end of the indicator as the true basophilic granules. As the cell matures, the chemical reaction shifts

gradually toward the alkaline end with a more striking change detectable between myelocyte and early leucocyte, or between early leucocyte and leucocyte of the blood. The deeper stain of the granules of the myelocyte makes it a conspicuous cell in supravital preparations, and the same is true with early leucocytes when many of them have been called into the blood. The staining of the granules of the leucocytes of the Arneth range grows fainter and fainter as the cell matures, until in the non-motile phase there is no reaction at all. Thus the changing chemistry of the granules is a gradual phenomenon, extending from myelocyte type A to the senile leucocyte, with one sharp drop during the early leucocytes. The granules of the mature leucocytes do not represent an exact point in pH, but rather they function within a certain range. Whether the neutral red is a more or less sensitive indicator of this change than the methylene-blue-azur, or whether it registers exactly the same change, one cannot say; but in fixed technique the process is also gradual, with a marked change in the group of the myelocytes type C. That there is no exact point for the striking change accounts for the lack of a consistent and precise definition of "promyelocyte." There are three elements that make it impossible to define the exact limits between promyelocyte and myelocyte; first, the estimation of the color change may vary with the observer; second, more important is the fact that the color of the granules in the fixed film varies markedly with the pH of the distilled water used in the technique; and third, probably different cells do not show the change at exactly the same point. It thus becomes necessary to estimate the definition of promyelocytes from the practical application in the counts in the literature. Schilling's definition is the most precise (125), and counts done in his laboratory (Yamamoto, 127) give for rabbit's marrow myelocytes 2 to 5 per cent, and promyelocytes 50 to 60 per cent. These figures indicate that promyelocyte may cover types A, B, and most of type C.

The changing chemistry of the granules is a fact, the physiological importance of which is at present probably underestimated; but as a basis of classification it fails to bring out the important fact that marrow functions from the mature levels. It has shown, it is true, that there is a shift to immature levels in pathological marrows, but it is not so sensitive an index of this change as the classification by the numbers of the granules.

Pattern of the red cells in bone marrow. A simplification of the study of red cells in marrow is involved in the work of Sabin (54) and Doan *et al.* (23), which led to the definition of the megaloblast as the earliest red

cell, the direct derivative of endothelium. The maturation of the red cell is shown in chart 2. The critical points are at the megaloblast and normoblast. The latter is the cell of common size after the last division of the erythroblast. The mitochondria reach their maximum at the stage of the early erythroblast, the largest type in the development of the red cells, which was shown as figure 45 by Doan *et al.* (23).

The data for calculating the proportions of the stages of nucleated red cells in marrow were given by Sabin and Doan in their table 3 (129).

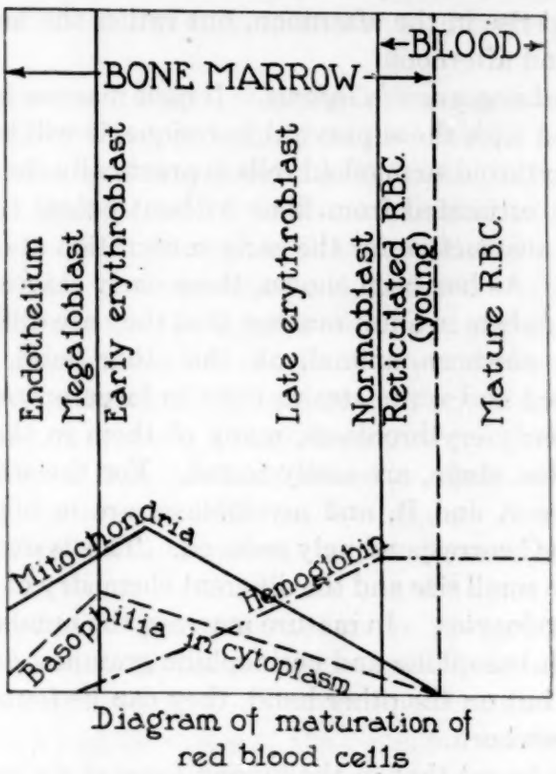

Chart 2

As for the white cells, the major supply of nucleated red cells is at the more mature levels; the normoblasts make an average of 69 per cent of nucleated red cells; erythroblasts, early and late together, make 30 per cent, and the megaloblasts were found in only two animals, 0.01 and 0.04 per cent. The early erythroblasts run about 1 to 4 per cent.

The clumps of nucleated red cells in normal marrow are made up of late erythroblasts and normoblasts, and the rhythmic delivery of the red cells lies in the reciprocal relationship of normoblasts and reticulated

red cells. Normal marrow does not draw upon endothelium and megaloblasts for its supply of red cells, and a simple anemia is represented in the marrow by an increase in the proportion of early erythroblasts. Only when a secondary anemia has become extreme is the pattern of the marrow thrown back to any extent to megaloblasts and endothelium. Sabin *et al.* (99) and Doan and Zerfas (128) have shown that the delivery of red cells from the marrow is rhythmic with a range of about 1,000,000 cells between the high and the low counts each day; as far as their observations have gone, the rhythm does not follow that of the white cells, with the marked rise in the afternoon, but rather the high points come both morning and afternoon.

Marrow in fetal stages and in infants. If bone marrow from a newborn rabbit be studied with the supravital technique, it will be seen that the proportion of erythroid to myeloid cells is practically the reverse of that of the adult, as estimated from films without actual counts. Such a marrow is most instructive for the early maturation stages of both red and white cells. As has been shown, these early stages are present in such minimal numbers in adult marrow that they are difficult to demonstrate. In the newborn animal, on the other hand, the immature stages of both red and white strains exist in large proportion. In the red series, the early erythroblasts, many of them in the exceptionally large types of that stage, are easily found. For the white series also, myelocytes types A and B, and myeloblasts are in high proportions, with myelocytes C correspondingly reduced. This is excellent material for observing the small size and the different chemistry of the granules of the primitive myelocytes. In mature marrow, the numbers of the early myelocytes, with basophilic and eosinophilic granules, are too minimal to be observed, but on the other hand, they can be found easily in the marrow of the newborn.

Horwitz (133) found that in the human fetus of six months, the red cells predominated, making $\frac{3}{4}$ of the total; at eight months $\frac{9}{10}$, while at term she found the two strains equal. Lossen (122) found more erythroblasts in the newborn and during the first month of life than in the adult. Latiner-Mayerhof (134) found the marrow of the fetus predominantly agranular, with granulocytes running from 2 to 22 per cent before birth; in two babies that died at birth, the myelocytes were low, but there were 31 and 44 per cent leucocytes in the marrow, corresponding to the known leucocytosis. This decrease of leucocytes in the marrow lasted only a day, while the leucocytosis of the blood, 10,000 to 22,000, with 81 per cent neutrophilic cells, persisted three days, indi-

cating redistribution phenomena. Her tables give a range of from 2 to 90 per cent of myeloid cells, which must be analyzed in terms of two different factors, namely, age given in months for the first year, and the varying diagnoses. Kramár and Heinsch (33) think that the marrow of the first year has 61 per cent granulocytes to 25 per cent lymphocytes, which is to be compared with the high percentage of lymphocytes, 27 to 44 per cent, the first eight years of life found by Doan and Zerfas (128). This point must be restudied with reference to the presence of lymph follicles in the marrow, and more especially in connection with the high relative percentage of lymphocytes to granulocytes in the blood of infants.

In earliest embryonic stages, only red cells are produced; during fetal stages, white cells are formed only in small numbers. It is clear, then, that there is a change in erythroid-myeloid ratio in the blood-forming organs, from a beginning of 100 to 0 to an adult ratio in bone marrow around 1 to 3. In the pattern of both erythroid and myeloid elements, the marrow of the newborn represents a marked shift to immature forms, as compared with the adult pattern, or, stated the other way, during development the pattern of cells of bone marrow shifts toward the right, from a predominance of the immature types to a marked predominance of the mature stages. Thus it is easy to understand both why the cells of the blood of the newborn are, as a whole, shifted to the left, and how immature marrow cells, as, for example, myelocytes B, may appear in the blood with the same ease as the metamyelocytes in the adult.

Megalocaryocytes in bone marrow. Besides the specific forerunners of the blood cells, two other types of cells are found in bone marrow, megalocaryocytes and clasmatocytes. The multinuclear giant cells, the osteoclasts, which lie along the edges of the bone, are not here considered as cells of marrow. The megalocaryocytes have been related to the formation of platelets ever since the demonstration by Wright (135) that they give off masses of cytoplasm which fragment into platelets. This process was watched by Sabin (95) in megalocaryocytes which found their way into the peripheral blood.

In the normal rabbit these giant cells exist in small percentage, about 1 per cent in the marrow. It was shown by Frank (136) that the giant cells of the marrow decrease in proportion to a fall of platelets. Doan and Sabin (85) followed the fall in platelets which accompanies an anemia and leucopenia from an invasion of the marrow by tubercular tissue; in the subsequent recovery of the marrow, the platelets always

started to rise first, then hemoglobin, then red cells, and finally the leucocytes. For every stage they killed a rabbit after counting the blood and found that degeneration and disappearance of megalocaryocysts paralleled the fall in platelets in the blood; and that, as the platelets rose, megalocaryocytes regenerated, first as small cells, with little cytoplasm in proportion to the nuclei. Finally, there were four or five in almost every field in contrast to the original number of one or two. Anselmino (137) found a decrease in megacaryocytes in the marrow associated with multiple hemorrhages in septic states.

Clasmatocytes. Clasmatocytes make also a small group of cells in normal marrow, running about 1 per cent in the rabbit. Doan and Zerfas (128) found them in the same per cent in normal human marrow. They exist in two forms, scattered cells and the adventitial types of Marchand. They were shown by injections of trypan blue by Evans (138) and with India ink by Wislocki (139); their numbers become increased in the chronic reaction to particulate matter by a stimulus to endothelium (Doan *et al.*, 23). In the stage of the resolving tuberculosis of the marrow in rabbits, Doan and Sabin (85) found that the blood vessels were marked out by lines of adventitial clasmatocytes filled with acid fast débris of the bacilli. In pernicious anemia, clasmatocytes increase in number, as shown by Doan (140), up to 10 per cent, and they become markedly phagocytic toward red blood cells, even engulfing the nucleated forms (Doan, 140; Peabody and Broun, 141).

Extramedullary formation of granulocytes and erythrocytes. If redistribution phenomena in the peripheral blood and an ectopic formation of bone marrow have been ruled out, the correlation between blood and bone marrow is quite exact. For example, in a series of tuberculous rabbits, of which repeated counts of the blood had been taken and charted, Doan and the writer made a correct estimation of the condition of the blood at the time of the death of the animal, from a study of the bone marrow alone in 66 out of 68 animals. Of the two in which the correlation was not made, one showed formation of red cells in the spleen, and so the regeneration of red cells was underestimated for the marrow, and in the other there had been too much autolysis of the marrow before fixation to make the study possible. It is well established that the formation of red cells or granulocytes may be easily shifted to the spleen, and this is thought to be explained by the fact that the formation of blood takes place normally in the spleen in late fetal stages. The formation of blood cells begins in the yolk sac; in the human embryo, it begins first in the body stalk and the more general connective tissues, and is at

the start wholly of red cells. The process then passes first to the liver, and then to the spleen, before the bone marrow functions.

Beside this phenomenon, which may be regarded as a return to fetal conditions, as recently indicated by Ballin and Morse (142), there are two other types of extramedullary formation of myeloid and erythroid elements. The presence of granulocytic myelocytes, frequently eosinophilic in type, diffusely scattered in connective tissues, is common in pathological conditions, both around chronic inflammatory areas and in the sepa of tumors (Weill, 143). Eosinophilic myelocytes are almost constantly present in the bronchial lymph glands of the pig, possibly correlated with the fact that the air passages practically always contain small, round worms. The question concerning the origin of such cells in the diffuse connective tissues is obscure. There are three possibilities: First, they might arise from the primitive cells along the blood vessels, in which case the stages of myeloblast, myelocytes A and B might occasionally be found. Second, they might arise from the division of myelocyte C from the bone marrow. Third, a number of recent observations tend toward the idea that cells now regarded as more differentiated types may give rise to granulocytes directly instead of by the process of maturation characteristic of bone marrow; for example, the view of Franco and Ferrata (144), and Ferrata (145) that the cell known as clasmatocyte, macrophage or reticulo-endothelial type, may elaborate specific granulations in its cytoplasm and become a granulocyte. They found such cells in the blood stream, containing either neutrophilic or eosinophilic granulations, and termed them "hemohistiocytes." These observations and interpretation have been confirmed by Richter (146) and by Jordan (147); but Doan and Sabin (148) have offered another explanation, namely, that neutrophilic and eosinophilic granules may be phagocytized from leucocytes and their débris, and that phagocytized fragments of red cells may be confused with true eosinophilic granules in fixed tissues.

Closely allied to Ferrata's view is the newer evidence from the method of tissue culture, which tends to show that the clasmatocytes and monocytes of the connective tissues and blood are the same cell under different phases of activity (Carrel and Ebeling, 149, 150; Lewis and Lewis, 151). Carrel and Rosenberger have shown by moving pictures of the living cells that both clasmatocytes and monocytes move by waving surface film, which is a type of motility quite different from that of leucocytes and lymphocytes. Carrel and Ebeling believe that one type can be changed into the other under the conditions of tissue culture

through the control of the nourishment of the cells. Lewis and Lewis have added that the epithelioid cell also belongs to the single strain of the phagocytic mononuclears, a view that has been confirmed by Gardner and Smith (152). Lewis (153) followed the transformation of granulocytes into macrophages in the living frog's lung. To these observations must be added the idea that mature lymphocytes of the tissues may elaborate the specific granulations and become leucocytes (Weidenreich, 154; Weill, 155, 156; Bloom, 157; Downey, 102). An analysis of the literature on this point is given by Downey. The occurrence of neutrophilic and eosinophilic myelocytes in the tissues abnormally must be studied in relation to the normal occurrence of histogenic basophiles. All these observations concern the relations of the mature or differentiated cells of the connective tissues to each other. On the functional side, Sabin, Doan and Cunningham (158), Cunningham *et al.* (88) and Sabin and Doan (159) regard the monocytes and the clasmatocytes as distinct strains and have shown that the epithelioid cell is derived from the monocyte. As an example of an important functional difference, Sabin and Doan have shown that the two cells react differently toward tubercle bacilli, inasmuch as this organism is to be found in the granular form in the clasmatocyte and in the rod form in the monocyte and epithelioid cell. The problem of the relationships of the cells of the connective tissues is very complex; it involves the consideration of both degeneration and de-differentiation *in vitro* as well as such possibilities *in vivo*. It is not possible at the present time to evaluate all the evidence concerning the reactions of the cells of the connective tissues to abnormal conditions; but whatever may prove to be the genetical relationships of the different types, it is probable that the differentiations of the cells of the connective tissues correspond to varying functional needs. These relationships of mature cells will only be analyzed through further chemical-biological studies, but this particular problem is not involved in the simpler processes of maturation of leucocytes in normal bone marrow. Ono (160) has brought about the extramedullary formation of both granulocytes and normoblasts by means of injections of red blood corpuscles from which the hemoglobin had been removed with distilled water. The condition, discussed later, involved a marked stimulus to erythrogenesis in the marrow.

Beside this diffuse reaction of granulocytic types in the connective tissues, ectopic bone marrow as a definite nodule has been frequently observed. Recent reports (Brannon, 161; Saleeby, 162; Lang, 163) give the literature. Such nodules have a definite vascular pattern; they

are frequently predominantly of red cells and have a pattern of definite collapsed capillaries, like that of gelatinous bone marrow. Bone marrow occurs also in cartilage usually wholly hyaline; it has been produced experimentally by slowing the blood flow (Sacerdotti and Frattin, 164; Maximow, 165).

Vasomotor reactions. With the concept in mind that bone marrow as an organ is the place where two types of cells are made, that it holds a large store of each of the two strains almost ready for delivery, and that its store of immature forms is small in numbers, but of exceedingly high potentiality toward multiplication, growth, and maturation, the question of the mechanism to maintain such a normal structure becomes the major problem of hematology.

The mechanism involves both vasomotor and chemical regulation. The newer work on the vascular system (Doan *et al.*, 23) shows that whatever controls the proportion of collapsed capillaries to open sinuses is an important factor in the regulation of the proportion of red cells to myeloid elements. Thus the problem of vasomotor control of marrow is of great significance. It is clear that there are dynamic factors due to the fact that the marrow is in a closed box; second, that vasomotor control by the action of nerves on the muscle of the arteries is important, and that all the factors in control of the closing of capillaries discussed by Krogh (5), Hooker (74), (75), Richards (77) and Rich (76) are met here with the further complication of the special dynamic factors. Thus, while increased and decreased blood flow through the vasomotor nerves of the arteries plays a part, it is not clear whether the collapsing of the capillaries is regulated in part by decreased blood flow and in part by a local chemical stimulus applied to endothelium. In marrow, the collapsed capillary gives areas of prolonged low oxygen tension. Beside the important factor that regulates the proportion of open to collapsed capillaries in active marrow, the mechanism that controls the long continued low blood supply in yellow marrow is also significant.

When the pattern of levels within each group is considered, two different processes must be analyzed: first, the mechanism of the delivery of cells to the circulation, and second, the mechanism of maturation. Concerning the mechanism for the delivery of cells into the circulation, the question of how great a part vasomotor influences play is uncertain. Vasomotor phenomena with reference to blood have been studied by Ribadeau-Dumas and Roussy (166), Glaser (167), (168), (169), Glaser and Buschmann (170), Müller (171) and Yamamoto (127). The response of the marrow to the nerve stimuli has not been sufficiently

controlled with reference to the normal rhythms of the blood cells and to the fact that the range of normal variation in a day is 100 per cent, as is shown when counts are taken every fifteen minutes for several hours (Sabin *et al.*, 99). Glaser and Buschmann (172) found a variation of 53 per cent in a day, of 76 per cent between different days, and finally of 90 per cent. The leucocytosis following adrenalin is lymphocytic rather than myeloid (Doan and Zerfas, 128). Ribadeau-Dumas and Roussy cut the nerves of the hind leg in a rabbit and found less output in response to collargol; Yamamoto cut the nerves of the leg in eight rabbits; after four days he gave injections of streptococci and studied the marrow three days later. He found a marked increase in the cells of the marrow and discovered that they were more mature in the leg without nerves. He concluded that while maturation is a chemical phenomenon, the calling of cells from the marrow is vasomotor. It is not likely, however, that such a separation of vasomotor and chemical factors can be made. For the admission of cells into the circulation two structures are involved: the endothelial walls of the sinuses and the blood cells themselves. A stretching of the wall of the sinusoids favors the entering of leucocytes between endothelial cells. This can be seen by watching the living vascular membranes from a chick of the sixth and seventh days of incubation; the young granulocytes exist there, as in adult marrow, not singly but in groups at the same stage of maturation; such a group of granulocytes can be seen to move *en masse* against the wall of the capillary until the wall is bent inward; when the stretching reaches a certain point a leucocyte close to the wall flows in between two endothelial cells and then the rest all follow in rapid succession. The same process of stretching of the walls of capillaries was shown in the early studies of inflammatory tissues to let the leucocytes out of the vessels.

For the red cells, it is easy to see that a constriction or shortening of the endothelial wall just at the point where a narrow neck connects the patent sinusoid with a hematogenic capillary distended with developing cells, would favor the opening of the capillaries. The mechanism of such a local lengthening and shortening of endothelial walls, whether passive or related to increased or decreased blood flow, or to local chemical stimuli for endothelium, has not been analyzed. However, the nice adjustment which results in the normal admission of only those cells that are functionally mature must also involve chemical stimuli that affect the blood cells directly only when they are mature. For the white cells such chemotactic factors have long been known.

Chemical factors. A leucocytosis involves both an increased delivery

of cells from marrow and an increased maturation of myelocytes B and finally of type A; these two reactions are to be expressed as the result of two different factors, a chemotactic factor (C) to account for the increased delivery of cells and a growth-stimulating or maturation factor (M) for the shift to the less mature cells in the marrow pattern. Muir (173) in 1898 analyzed leucocytosis as depending on two processes but thought of them as due to the same substance.

If, on the other hand, inactivated typhoid bacilli be administered to a rabbit and the blood be followed through the stage of the leucocytosis, the marrow will finally be found entirely depleted of both myelocytes C and B and reduced to the low level of myelocytes A and myeloblasts (Doan *et al.*, 23). Such a marrow corresponds to a state of leucopenia, and the experiment can be expressed as representing a condition due to a chemotactic factor minus a maturation element.

This concept of leucocytosis and leucopenia postulates the infecting organism as introducing both a chemotactic and a maturation factor when a leucocytosis results and a chemotactic factor alone when a leucopenia follows a temporary leucocytosis. The question may be raised as to whether a leucopenia may not be due rather to a depressant factor than to the absence of a stimulating substance. In the experiments cited with inactivated typhoid bacilli, the cells in the marrow showed no toxic effect and the marrow readily regenerates after the experiment. In typhoid fever itself a totally different element is introduced as shown by Quincke and Stühlen (45), Fraenkel (46), (47) and Hartwich (48), because the marrow is frequently infected locally, with abscesses, necrosis and hemorrhage.

Knowledge of chemotactic reactions on blood cells goes back to Cohnheim and to Ehrlich. The existence of such substances in bacterial proteins was shown by Buchner (174) who obtained albuminates from the Friedländer bacillus and showed that they produce pus. Roemer (175), (176) followed the development of a leucocytosis produced with the bacterial protein of Buchner and with Koch's tuberculin as chemotactic factors. The first suggestion of such a chemotactic factor produced by the body itself was by Horbaczewski (177) who thought that digestion leucocytosis was correlated with an increase in uric acid in the blood. It is now known, however, that digestion leucocytosis does not exist, for the afternoon rise in leucocytes has been shown to take place whether food is taken or not (99).

Of the many substances that have been shown to call leucocytes from marrow, nucleic acid is most likely to be a part of the normal mechanism.

Löwit (178) found a leucopenia followed by a leucocytosis after a large number of substances, bacterial proteins, hemialbumose, peptone, pepsin, and nucleic acid and nuclein; he also included from the literature blood extracts, curare, uric acid and sodium urate. He thought that the leucopenia was due to a destruction of the leucocytes and that the leucocytosis was the reaction of the marrow to replace them. Thus his theory was that leucolysis and leucocytosis were cause and effect. Goldscheider and Jacobs (117) repeated the experiments and regarded the phenomenon as due to chemotaxis. They brought forward the view that the leucopenia which is the initial reaction to hemialbumoses, nucleic acid, tuberculin, bacterial proteins, and organ extracts is due especially to a lodging of leucocytes in the lung. Thus they introduced the important idea that the analysis of a chemotactic action on the marrow depends on the understanding of peripheral redistribution phenomena in order to distinguish exactly how much of an observed effect on the blood cells is due to action on bone marrow.

The most recent contribution on this subject is that of Doan, Zerfas, Warren and Ames (132) in a study of the effect of large doses of nucleic acid. They have demonstrated that the phenomenon of peripheral redistribution of blood cells can only be studied in the living animal. With the group of four workers they made synchronous repeated counts of the blood from a peripheral vein, the heart, the liver, and the spleen. The animal was kept anesthetized for hours under sodium barbital. From the liver and the spleen they cut tiny bits of the tissue for histological control at the time of each count. In the study of the leucopenic phase, the counts of blood from three places, peripheral vein, heart, and liver, ran parallel, all showing the leucopenia, while there was steady increase in the percentage of granulocytes in the spleen, correlated with an increase in the size of this organ, as determined by the oncometer. The piling up of granulocytes in the spleen is also shown in their photographs.

That the spleen was the only organ involved in the leucopenia was demonstrated by the complete elimination of this phenomenon after splenectomy. After making a long series of counts of the blood from the liver, they repeatedly showed that the only striking change in the white cells in that organ came at the moment of the death of the animal; moreover, the change, starting as soon as the punctures could be made after death, consisted only in an increase in the total number of white cells per cubic millimeter with no change whatever in the proportions of the different types of white cells. It is thus clear that all studies of redistribution phenomena must be made on the living animal with both total and differential counts.

On the other hand, their studies of the bone marrow showed clearly a chemotactic effect of the nucleic acid, with the massing of leucocytes around the patent sinusoids, a marked diapedesis into the vessels, and the vacant areas of the marrow from which the granulocytes had been drawn. The increase of the granulocytes in the blood stream was temporarily masked by their storage in the spleen, with the resulting leucopenia. They then found that the granulocytes could be called from the marrow by the split products of nucleic acid, adenin, and guanin nucleotide, and that after these substances the leucocytes were not withdrawn from the circulation by the spleen so that there was a direct leucocytosis without the temporary leucopenia. It is thus likely that nucleic acid and its derivatives are important physiological factors in the chemotactic reaction, and that the showers of non-motile leucocytes in the circulating blood may give a rhythmic discharge of such products into the circulation. It will be interesting if the rhythmic extrusion of the nuclei of normoblasts plays a rôle in this phenomenon.

Thus there are many observations of chemotactic substances and quite definite evidence concerning their physiological mechanism. Of maturation factors there is but meager knowledge. In this connection it is well to make clear that three processes are involved in the maturing of the marrow cells: first, cell division; second, the growth of cells between the cycles of division; and third, the varying factors in the cytoplasm which are represented on charts 1 and 2, such as the elaboration of specific granules, or hemoglobin, and the decrease of other substances built up in the earlier stages. To these must be added nuclear changes such as the loss of nucleoli in the more mature stages and the increase in the amount of chromatin. The physiological maturation factors for white cells are not known, but we have postulated that those bacteria that produce a sustained leucocytosis introduce such a factor, for they produce an increased division, growth, and maturing of the less mature myelocytes in the marrow far beyond the normal amount. Bacon, Novy and Eppler (179), while not discriminating between chemotactic and maturation factors, consider that even in infections the stimulus to an increased activity of the marrow comes from altered body proteins. They have studied dehydration phenomena from the injection of hypertonic solutions and postulate that the resulting fever and leucocytosis are like the reactions of infections and due to changes in the body proteins from the hydration. Thus they consider that the mechanism of the leucocytosis in an infection is due to an increase of the substances in the body that afford the normal stimulus, rather than to some substance

from the bacteria. The relationship of the degree of the leucocytosis to the resistance of the animal in infections has been repeatedly confirmed since Metchnikoff; and thus, as long as none of the substances involved in these reactions is known, variations in response of the animal must be studied in terms of the amount of the infection and possibly differences in the power of the hematopoietic tissues to respond.

The only knowledge of maturation factors concerns red cells. The recent application of the liver diet to pernicious anemia (Minot and Murphy, 68, 69) and the isolation from liver of the specific substance (Cohn *et al.*, 180) involve the discovery of a maturation factor. It has long been known that megaloblasts were to be found in increased numbers in the marrow in pernicious anemia, but only the recent studies with material taken at biopsy (Zadek, 181; Peabody, 34) have made it clear that in the height of a relapse the bone marrow of pernicious anemia may be defined as exhibiting a condition in which the maturation of the red cells has been arrested in the megaloblastic phase. In terms of the normal pattern of erythrogenesis, the marrow has been changed from the normal predominance of normoblasts, 69 per cent, to a much greater predominance of megaloblasts. However, the condition is not only an extreme shift to the left in erythrogenesis in the marrow, but there is also disorder of maturation with the disappearance of the mechanism for the reduction of the red cells to a standard size. The second line on chart 2 has to be erased to express the condition. In the peripheral blood the red cells normally continue to grow smaller by fragmentation, a process markedly increased in secondary anemia (Rous and Robertson, 182, 183; Doan and Sabin, 148), but cells larger than normal can only come from disorganized maturation. Minot and Murphy (68), (69) have shown that by the liver diet the normal mechanism of maturation is restored by the speedy appearance in the peripheral blood of reticulated red cells of normal size.

The recent discovery by Welo and Baudisch (184), (185) of "active iron," which is a ferromagnetic cubic form of iron oxide reopens the whole subject of the effect of iron on erythrogenesis. It has already been used successfully in the treatment of anemias of children by Moldawsky (186) and must be studied experimentally in its action on bone marrow. The work of Ono (160) is likewise important. He took blood, washed the corpuscles repeatedly with salt solution to free them from serum, pipetted off the leucocytes and then shook the red corpuscles with distilled water until completely hemolyzed. He found that the corpuscles freed of the iron content were a marked stimulus for erythro-

poiesis in rabbits when given in medium doses, 2 to 3 cc. per kilo for autologous corpuscles, 3 to 8 cc. for isologous and 8 to 30 cc. per kilo for heterologous cells. These injections were repeated every day, or every other day until a certain amount had been given. Small doses were ineffective and large doses toxic. The medium doses corresponded to the estimated physiological destruction of red cells per day. There was an increase in red cells, with the reticulated forms, reaching a maximum in from 2 to 7 hours, with a fall to the original level and a subsequent rise in 2 to 3 days which was sustained for a few weeks. In the tables the change was about a million cells during the sustained rise. Controls were made with hematin and extracts from lymph glands, muscle, and kidney without the specific effect on erythrogenesis.

From the preceding pages the following points concerning the structure of bone marrow as an organ can be summarized. There is a normal proportion of erythroid to myeloid activity for the adult, which is the reverse of that at the time of birth. There is a normal pattern of maturation in each group, erythroid and myeloid. For each group the major supply of cells is at a stage near maturity, with the more primitive stages represented by minimal numbers. The delivery of cells to the circulation is from a reciprocal relationship between the late stages of marrow cells and the early phases of the blood cells. The patterns of marrow and the rhythmic delivery of cells into the circulation depend on the fact that maturation and cell division in marrow occur in rhythmic, orderly cycles. Blood formation begins in the earliest embryological stages with red cells alone; white cells are in minimal numbers in fetal stages; the pattern of marrow at birth shows a predominance of the early phases of each strain the reverse of the adult pattern, and the maturing of bone marrow involves a gradual shifting of the entire pattern to the stage of a predominance of the more mature marrow cells. Most pathological changes in marrow involve a shifting back toward the left from the adult pattern. Whenever the function of bone marrow has been depressed in both red and white strains, the onset of recovery is first in erythrogenesis, the red cell being of more fundamental importance to the organism. Normal maturation involves giving to the blood both red and white cells of a uniform size, since neither definitive red cells nor granulocytes divide, and in a relatively uniform state of maturity.

The earlier discussions concerning monophyletism, dualism, and polyphyletism were concentrated on the problem of exactly where in the cells of level I, table 1, came the irreversible determination into one of the

three strains, the granulocyte, lymphocyte, or monocyte. The modern aspect of this problem can be stated as follows: In bone marrow the normal mechanism is adapted to the production of three strains of cells, red cells, granulocytes, and megacaryocytes for the platelets. Collapsed capillaries make zones of low oxygen tension which favor red cells; widely dilated sinuses give abundant and slow blood flow which brings the maturation factors in the right concentration for granulocytes between these vessels. In lymph glands the abundant and slowly flowing lymph of the lymphatic sinuses brings the maturation factors in the right concentration to produce lymphocytes. Monocytes are produced in small numbers in the diffuse connective tissues. These are the normal reactions, but bone marrow may be thrown over to the production of almost no white cells but lymphocytes in lymphatic leucemia, or almost no cells but monocytes in massive tuberculosis of the organ. Granulocytes or monocytes may be formed in lymph glands, and granulocytes, lymphocytes, or monocytes may be produced in the connective tissues. The modern aspect of the problem involves a shift in the emphasis from the morphological question to the investigation of the chemical maturation factors which determine these three strains of cells. These chemical factors are to be sought through the chemical analysis of bacteria known to affect blood cells, in certain organ extracts, as well as in specific dietary factors, some of which may be vitamines (Whipple *et al.*, 66, 67; Minot and Murphy, 68, 69; Drew and Mottram, 187; Koessler and Maurer, 188).

Pathological conditions in bone marrow can be divided into three groups. First, the conditions of simple leucocytosis and leucopenia together with secondary anemia and aplasia, in which the normal pattern of marrow has been shifted toward the less mature stages, with or without increased maturation from the immature cells. This condition involves no deviation from the normal rhythmic division and maturation. Second, states like pernicious anemia and leucemia, in which the pattern of the marrow has also shifted toward the immature states, even to the most immature, myeloblasts and megaloblasts, but with complete disorder in cell division and in maturation, so that immature cells of all sizes and stages of development come to the blood stream. Third, conditions in which there has been invasion of the marrow by infections or with new growth of tissues involving such phenomena as replacement, necrosis and hemorrhage, rather than disorders involving only the maturation patterns.

With such an analysis, it is evident that a start has already been

made in a new type of hematological research in which the goal is to find the chemical substances which affect the endothelium of sinusoids and capillaries, the primitive mesenchyme and the marrow cells, both red and white, and thus regulate the normal mechanism of bone marrow as an organ. Such knowledge would make a basis for unraveling the conditions which underlie abnormal changes in the mechanism.

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V. Bone Marrow as an Organ

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THE AEROBIC OXIDASES

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At the beginning of the present century it was generally believed that oxidation processes in living organisms were brought about principally by catalysts which were able to activate oxygen. This may be traced to the happenings of the time. Bertrand (8), (9) had published his investigations on laccase, the oxidising system of Rhus vernicifera, and along with Bourquelot (16) had shown the existence in fungi of an enzyme tyrosinase, so named because of its oxidising action on the amino acid tyrosine. In addition, the enzymes peroxidase and catalase had been clearly distinguished and separated by Löw (45). These enzymes revealed their action only in the presence of oxygen or peroxides, and this fitted in with the current views of Bach, Engler and Wild, on the activation of oxygen during oxidation processes. An excellent account of the opinions held up to the year 1910 is given by Kastle (42) in his monograph on *The Oxidases.*

During the last fifteen years the position has changed, largely as a result of the new views put forward by Wieland (73), (74) and the extended investigations of Thunberg (68). Both these observers regard the oxidation of many organic substances and particularly those of biological importance as being essentially a removal of hydrogen. If the oxidation process results in the final addition of oxygen to the molecule of the substance oxidised, this is added not directly as atomic or molecular oxygen, but in the form of the elements of water. The net result of this change, whether the addition of water precedes or follows the removal of hydrogen, is that oxygen remains in the oxidised molecule. By way of illustration a simple instance may be given. Acetaldehyde by addition of water forms a hydrate. If two atoms of hydrogen are removed from this, acetic acid is formed:

$$CH_3 \cdot CHO + H_2O \rightarrow CH_3 \cdot CH(OH)_2 \rightarrow CH_3 \cdot COOH + H_2$$

In order that this reaction may proceed, a substance must be present which will take up the hydrogen (the hydrogen acceptor), and for this

purpose, atmospheric oxygen or some readily reducible substance will serve. Colloidal palladium also will act as a hydrogen acceptor, but unless the hydrogen is in turn removed from the palladium the reaction does not proceed very far. If, in addition to the palladium, oxygen or an easily reducible dye such as methylene blue be added, then the reaction proceeds to completion, provided enough oxygen or dye be present. The fact that methylene blue is able to replace oxygen and that the aldehyde is none the less oxidised to acetic acid, shows that oxidation of the aldehyde can take place in the absence of free oxygen provided that a catalyst and a reducible substance are present. In this reaction, palladium is the catalyst, since the aldehyde is not oxidised or the methylene blue reduced unless palladium is added to the system. It may be regarded as activating and transporting the hydrogen from the donator (the aldehyde hydrate) to the acceptor (methylene blue.) According to Thunberg, oxidising enzymes which act in the same way as palladium are transporters of activated hydrogen, and they will act only in the presence of a suitable donator (the oxidisable substance), in which the hydrogen becomes activated, and a suitable acceptor, which the activated hydrogen reduces.

The presence of enzymes of this kind in living tissues has now been amply demonstrated, and they occur very widely. The practical test by which they are detected is that in the absence of oxygen they will bring about the oxidation of a suitable substrate, provided an appropriate hydrogen acceptor, e.g., methylene blue, is present. An oxidation catalyst of a similar type, but thermostable, is the component of muscle and other tissues which, in the presence of glutathione,will reduce methylene blue (Hopkins and Dixon, 41). One of the chief distinguishing characteristics of these oxidases, therefore, is that they can bring about oxidations in the absence of oxygen. They are usually described as oxido-reductases or dehydrogenases. On the other hand, several of the oxidising enzymes which were known prior to these, can, so far as is known, exert their action only in the presence of oxygen or of a peroxide, the oxygen of which they appear to activate. They represent a different type of oxidation catalyst, and for convenience may be classified as *aerobic oxidases*. Until recently it appeared as if these two types of oxidation system bore no relation to each other, but observations have been brought forward by Thurlow (69) which indicate that at least some of them may interact in a way which was not previously imagined.

Our knowledge of the oxidation systems of the cell is still in a relatively

primitive state, and it may well be that the distinction drawn between these two groups of enzymes, which is based on such information as to their action which we possess at the present time, will not persist in the future. It may be justified, however, on the ground that it is founded on good experimental evidence and is therefore of value as a basis for further enquiry. In support of this grouping of the oxidases it may be pointed out that Wieland (74), although able to demonstrate that palladium acting as a catalyst in the presence of a suitable hydrogen acceptor brings about several oxidations which are known to occur in living tissues, did not succeed in oxidising tyrosine by this method, although this amino acid is readily oxidised by the aerobic oxidising enzyme, tyrosinase. It must be borne in mind, however, that the failure to imitate *in vitro* the action of the aerobic oxidases may be due to failure to find the right hydrogen acceptor. Important experimental work which bears on this question has been carried out by Dixon (22), who has examined a series of possible hydrogen donators and acceptors for the xanthine oxidase of milk. He found that whereas the hydrogen donators were limited to hypoxanthine, xanthine, aldehydes and possibly adenine, the hydrogen acceptors covered a wide range of substances, including, among others, methylene blue, oxygen, hydrogen peroxide, Clark's reduction-potential indicators, iodine and benzoquinone. This does not support the idea of specificity of the hydrogen acceptor in oxidation-reduction reactions. It suggests rather, that failure to find an appropriate hydrogen acceptor which can replace oxygen in oxidations brought about by the aerobic oxidases, indicates that the mode of action of the enzymes in the two groups is different. By this it is not meant that the oxidations brought about by aerobic oxidases are not dehydrogenations. It seems likely that they are, at least in some instances. When this happens, the aerobic oxidases, combined with oxygen or a peroxide, must be presumed to act as a hydrogen acceptor, as for instance in the oxidation of quinol to parabenzoquinone by peroxidase and hydrogen peroxide. The subject will be dealt with later in considering the mode of action of the aerobic oxidases.

In order to accomplish most simply the purpose of this article, which is to review our knowledge of the aerobic oxidases in the light of experiments which have been published during the last few years, each of the enzymes will be dealt with in turn.

Peroxidase. The discovery of peroxidases was made by Schönbein (65) who, in his studies of ozone and peroxides, described the occurrence of ferment-like substances in plants and animals, which, like ferrous

salts, activate hydrogen peroxide or peroxides which are formed by autoxidation in the air. He used guaiacum tincture as one of the reagents for detecting peroxidases, but was not aware that catalase and peroxidase were different enzymes. This differentiation was first satisfactorily accomplished by Löw (45). The name peroxidase was first coined by Linossier, who obtained the enzyme from pus cells.

Peroxidases occur very widely in both animal and vegetable kingdoms. Often they are present along with other oxidising enzymes, but sometimes, in certain plants, e.g., roots of horseradish, they are not accompanied by other aerobic oxidases. They are therefore most readily prepared from such sources and their properties examined. When it is certain that other aerobic oxidases are not present, a relatively simple purification is all that is necessary in order to demonstrate their chief properties. A detailed study of the nature of the peroxidase of horseradish has been made by Willstätter and his collaborators (76), (77), (79), (80), (81). It was in the course of this investigation that the method of separation of enzymes by adsorption on appropriate colloids, which has added greatly to our knowledge about them, was evolved. The purification of the peroxidase from horseradish was accomplished by a series of adsorptions on alumina and kaolin from 50 per cent alcohol.

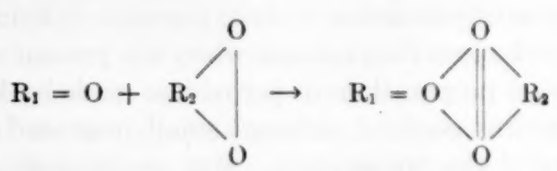
By means of this method, together with precipitation by tannin, extremely active products have been obtained. The activity is determined by a modification of Bach and Chodat's method. The amount of purpurogallin produced by the enzyme when acting upon pyrogallol under specified conditions as to temperature, concentration of pyrogallol and hydrogen peroxide, and reaction of the medium, is determined. The "purpurogallin number," which increases with the purity of the enzyme, is the amount of purpurogallin in milligrams produced by 1 mgm. of the enzyme in 5 minutes under the specified conditions. The purest product obtained had a purpurogallin number of 4,900, whereas that of the original horseradish was 0.25. In the preparations obtained in the earlier experiments the enzyme was accompanied by a nitrogenous glucoside, but this was not present when better methods of purification had been discovered. The most active preparation did not give protein or carbohydrate reactions, and the pentose found in earlier and less pure products had disappeared. It did not give a pyrrole reaction. It had a bright reddish-brown colour reminiscent of the porphyrins. Willstätter believes that the enzyme is coloured, and that the colour, therefore, of his final preparation is not due solely to impurities. The

colour remains when the enzyme has been inactivated, but this is probably due to its products of decomposition being also coloured. The enzyme when freed from most of its impurities is unstable, especially in aqueous solutions. Less pure preparations in which a glucoside is still present are more stable. The part played by iron as an oxidation catalyst is a matter of great interest and although the general question of its importance is outside the scope of this review it is necessary to consider briefly the relation of iron to peroxidase activity. It is well known that iron salts will cause an activation of hydrogen peroxide, and it has often been suggested that peroxidases possess this same property because of the iron which they contain. The fact that blood pigments and their derivatives which contain iron act like peroxidases has been held to support this view. That this may be due not to the iron alone is suggested by the results of an investigation by Robinson (61) of the rate of oxidation of linseed oil in the presence of hemoglobin, methemoglobin and hemin. The catalytic action of these pigments is much greater than that exercised by the iron they contain when it is present as an inorganic salt. In the earlier preparations of peroxidase made by Willstätter and his colleagues, the iron content, although small, increased approximately with the activity of the preparation. But products obtained later by the tannin method contained only small traces (0.04 per cent) of iron, although the activity had risen considerably. This therefore does not give much ground for the belief that the iron content and the activity of the peroxidase bear any relation to each other. But it does not solve the final question as to whether peroxidases would display their action even if they contained no iron, because iron-free peroxidase has not yet been obtained. It may be that iron is essential, but only minute amounts are necessary, or that peroxidase without iron would still be active and that the activity is increased by the presence of iron, much as iron catalyses the autoxidation of cysteine and of glutathione (Harrison, 38). The results of Willstätter and his colleagues seem to suggest that the latter view is probably a nearer approach to the truth than the former.

Peroxidase from horseradish acts best in a slightly acid medium. According to the recent work of Ucko and Banshi (70), the optimum pH varies slightly with the substrate. With guaiacol the optimum lies between pH 4.5 and 6.5, but with o.cresol it is between 3.5 and 5.0. With pyrogallol no optimum is measurable on account of the autoxidation of this polyphenol which occurs as pH 7.0 is approached.

The nature of peroxidase is not known. Willstätter and Pollinger,

(79) have published elementary analyses of their purest products which contained about 7 per cent of ash, and (reckoned on the ash-free product) 45.97 to 49.41 per cent carbon, 6.9 to 8.6 per cent hydrogen and 9.37 to 13.57 per cent nitrogen. This, however, gives no clue to their identity. Kastle and Loevenhart (43) have explained the action of substances such as peroxidase which activate hydrogen peroxide, as due to the formation of higher oxides which are unstable and easily lose oxygen if a suitable reducing substance is present. It is therefore of interest that Gallagher (30) has made the observation that the monoperoxides or ozonides of aliphatic aldehydes will act like peroxidases in the guaiacum reaction although they have no direct action on guaiaconic acid themselves. They appear to be formed by autoxidation of these aldehydes in the air. Gallagher suggests that in the reaction with hydrogen peroxide an unstable additive compound is formed and that this is the active oxidising agent.



Ozonide Peroxide Unstable higher oxide

Although Gallagher has shown that relatively crude peroxidase preparations give aldehyde reactions, this, as yet, has not been confirmed with the purer products obtained by Willstätter's method. Quantitative experiments made by Bach and Chodat many years ago on the oxidation of pyrogallol by peroxidase showed that excess of hydrogen peroxide inhibited the reaction. They believed that the peroxidase was destroyed. It has been shown recently by Willstätter and Weber (80), however, that the inhibition is due to the combination of peroxide with the enzyme. Willstätter (77) has obtained evidence which leads him to suggest that peroxidase acts by forming a compound with hydrogen peroxide in which the oxygen is more active than in the peroxide itself. Another compound is also formed in which the oxygen is no longer active, hence the inhibition with larger amounts of peroxide, which disappears if the amount of substrate be increased or if catalase be added. It is suggested that the active addition compound corresponds to the formula HO−OH and the inactive one to $H_2O=O$. When either of these gives up its active oxygen a hydrate of peroxidase is possibly formed, which again reacts with peroxide to give the active or the inactive addition compounds.

For the detection of peroxidases a considerable number of substances have been used. Many of these show no particular advantage when compared one with another. The commonest is tincture of guaiacum, or guaiaconic acid, which is the constituent of guaiacum resin responsible for the reaction. This is turned blue by peroxidase in the presence of a peroxide. The use of the guaiacum reaction, however, has given rise to so much uncertainty in investigations of the aerobic oxidases that it would be advisable to discontinue its use altogether. Unless the guaiacum tincture be fresh it may contain peroxides produced by autoxidation, and lead to confusion of peroxidases with the so-called "direct oxidases" which turn guaiacum blue without addition of a peroxide. This difficulty may be overcome by using guaiaconic acid.

Amongst other reagents which have been used for the detection of peroxidases are: benzidine, guaiacol, o-phenylenediamine, quinol, pyrogallol, a mixture of p-phenylenediamine and α-naphthol, phenolphthalin, o-, m- and p-cresol, leuco-malachite green and hydriodic acid. With most of these, either an intense colour or a precipitate is formed in presence of peroxidase and hydrogen peroxide, so that they lend themselves readily to the detection of the enzyme. The best reagents to use, however, are those which are either not attacked by other aerobic oxidases or are oxidised by them in a different way. For this reason, Chodat (7) has recommended the use of o-, m- and p- cresol.

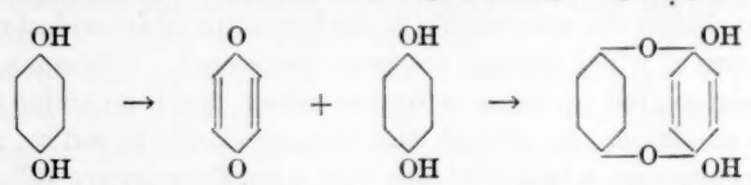
A dilute solution (0.1 per cent) of ortho-cresol gives with peroxidase in presence of hydrogen peroxide a green colour (brown in stronger solutions). Tyrosinase has no action on o-cresol; laccase produces the same colour as peroxidase but without the addition of peroxide. With p-cresol, peroxidase and hydrogen peroxide give a gradually developing milky turbidity due presumably to the formation of an oxidation product the nature of which has not yet been discovered. Tyrosinase also oxidises p-cresol, but produces an orange colour, and if an amino acid, e.g., glycine or alanine, be present, this changes finally to red or, according to the conditions, a beautiful blue with a red fluorescence (Chodat, 19). Laccase again gives the same reaction as peroxidase, but without the addition of hydrogen peroxide. Guaiacol also may be used for differentiating these enzymes. With peroxidase and peroxide it rapidly gives a deep red colour (Chodat). Tyrosinase has no action on guaiacol, and laccase produces the same action as peroxidase but without addition of peroxide. Peroxidases will, however, act on other substances besides phenols, aromatic diamines and their derivatives. Nitrites are oxidised to nitrates, for instance, by the peroxidase of milk (Thurlow,

69), and it has been shown by Harrison and Thurlow (39) that when hypoxanthine or xanthine is oxidised by the xanthine oxidase of milk in the presence of oxygen, the hydrogen peroxide produced as a result of oxygen acting as a hydrogen acceptor can serve to bring about other oxidations in the presence of peroxidase, e.g., nitrites to nitrates, or the oxidation of an ether-soluble constituent of the milk, probably an unsaturated fat.

These results indicate how an oxidation brought about by an oxido-reductase in the presence of oxygen may be the cause of a secondary oxidation in the presence of peroxidase, because hydrogen peroxide is produced by the oxido-reductase system. Under similar conditions, the secondary oxidation of lactic acid and β-hydroxybutyric acid are not brought about by peroxidase, but they do occur in the presence of a ferrous salt. Secondary oxidations of this type due to the association of peroxidase with an oxido-reductase are possibly of importance in the body, but so far the substrates examined have been few in number, so that an extension of this work to other substances of biological importance would be valuable.

The oxidation products produced by peroxidase, when acting on various substrates in the presence of hydrogen peroxide have been investigated only in part.

The nature of the substances produced from o-, m-, and p-cresol is not known. Their colour and failure to react with aniline[1] suggest that they are not quinones, which is of particular interest since there is no doubt that peroxidase produces quinones from polyphenols. Quinol, for instance, has long been known to yield p-benzoquinone, which then combines with excess of the quinol to give quinhydrone.

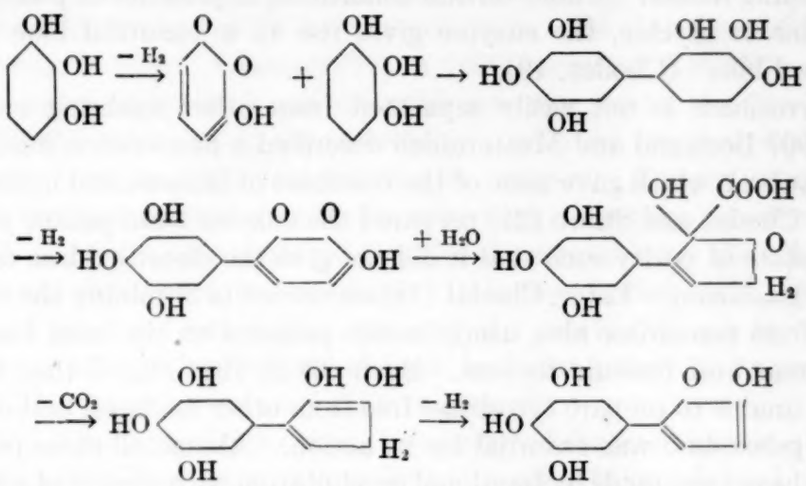


Quinol p-Benzoquinone Quinhydrone

Similarly, it has been shown recently by Pugh and Raper (55) that catechol and homocatechol give rise to ortho-benzoquinone and 3:4-toluquinone respectively, which may be separated from the reaction mixture in the form of their anilino compounds by acting on catechol or homocatechol with peroxidase and hydrogen peroxide in the presence

[1] Author's unpublished observations.

of aniline. The formation of purpurogallin from pyrogallol is preceded, according to Willstätter and Heiss (78), by the formation of 3:hydroxy-o-benzoquinone which then condenses with a second molecule of pyrogallol to form a hexahydroxydiphenyl. This by oxidation is supposed to yield an ortho-quinone, which undergoes rearrangement with the formation of a cyclo-pentenone derivative. By loss of CO_2 from this, and subsequent oxidation, purpurogallin is formed. These changes are represented as follows:



Purpurogallin

There is no doubt that a further study of the chemical action of peroxidase and peroxide on phenolic substances is very desirable.

Tyrosinase. This enzyme was first discovered by Bourquelot and Bertrand in 1895. They recognised that a substance present in Russula nigricans was turned black by an enzyme present in the same fungus, but was unaffected by the enzyme laccase from the lac tree. It was therefore a new enzyme. The next year Bertrand identified the chromogen from Russula nigricans as the amino acid tyrosine, and Bourquelot (15) then named the enzyme tyrosinase. Bourquelot described eight groups of fungi in which he found the enzyme, and mentioned the fact that these fungi also turned guaiacum tincture blue without the addition of hydrogen peroxide. Tyrosinase has since been found to be widely distributed in the vegetable kingdom and to be present in many invertebrate animals. The plant or animal juices which contain it usually darken on exposure to air, due to the presence along with the enzyme either of tyrosine or of some other substrate on which the enzyme acts.

Many such substrates have been described, and a useful summary of them is made by Chodat (18).

The characteristic property of the enzyme is that in presence of air it changes tyrosine solutions to red, then reddish-brown and finally black. An insoluble black pigment, melanin, is eventually deposited. It also gives yellow, red or reddish-brown colours with various mono- and polyhydric phenols. When amino acids are present the colour produced by the action of the enzyme on the phenol is modified, usually becoming redder. Under certain conditions, in presence of p-cresol and alanine or glycine, the enzyme gives rise to a beautiful blue colour, "cresol blue" (Chodat, 19).

Tyrosinase is not easily separated from other oxidising enzymes. In 1907 Bertrand and Muttermilch described a preparation made from wheat bran which gave none of the reactions of laccase, and in the same year Chodat and Staub (21) prepared the enzyme from potato peelings in a state of purity such that it did not give the direct oxidase reaction with guaiacum. Later, Chodat (18) succeeded in obtaining the enzyme free from peroxidase also, using for this preparation the fungi Lactarius vellereus and Russula foetens. Bach (6) in 1914 stated that he had been unable to prepare tyrosinase free from other oxidases, and claimed that peroxidase was essential for its action. Almost all these preparations have been made by fractional precipitation with alcohol of solutions containing the enzyme. Tyrosinase is usually precipitated before peroxidase, and the purest preparations have been obtained by redissolving the enzyme and reprecipitating it with alcohol, the process being repeated several times. Positive evidence of the absence of other enzymes from these pure preparations is of considerable value when the nature of the enzyme is under consideration, and it appears from Chodat's results that tyrosinase is an individual enzyme which does not depend for its action on peroxidase. It cannot therefore come into the class of "direct oxidases" which were described by Bach and Chodat (4) in 1903 as consisting of an enzyme-like substance which yields a peroxide on exposure to air (oxygenase) together with a peroxidase. The difficulty of separating tyrosinase from other oxidising enzymes, and the failure to appreciate the somewhat narrow limits of hydrogen ion concentration within which it acts, are no doubt responsible for some of the obscurity hitherto surrounding it.

The nature of the action of tyrosinase both on the amino acid tyrosine and on the phenolic substances on which it acts has given rise to a considerable amount of experimental work. Until recently this

has been concerned principally with attempts to explain the series of changes which are produced when the enzyme oxidises the amino acid tyrosine with the final production of melanin. The first suggestion, made in 1900 by Gonnermann (32), was that tyrosinase is a hydrolytic enzyme which splits tyrosine into substances that oxidise easily in the air, and homogentisic acid (2:5 dihydroxyphenylacetic acid) is mentioned by him as the most likely precursor of melanin. Bach (5) in 1909 showed that when tyrosine was submitted to the action of tyrosinase in an atmosphere of hydrogen, no easily oxidisable products were produced and no change could be detected in it. He therefore rejected Gonnermann's view. Gessard (31) in 1900 suggested that the action of the enzyme on tyrosine was separable into two stages, the first consisting in the oxidation of the tyrosine to yield a red pigment, and the second the "condensation" of this to give melanin. Gessard regarded the first reaction as analogous to the reddening of tyrosine solutions by Millon's reagent, and considered that for the second stage the mineral salts in the tyrosinase are essential. It is now known that the red substance produced in the first stages of the reaction is not identical with that produced by Millon's reagent, and that mineral salts are not essential for the subsequent change to melanin.

In 1913, Chodat and Schweizer (10) described the deaminising action of tyrosinase. They were able to detect traces of ammonia and of the corresponding aldehydes when tyrosinase acted on certain amino acids in the presence of p-cresol. Bach (6) in the following year then suggested that tyrosinase is a mixture of enzymes, a deaminase causing deamination of tyrosine to yield p-hydroxyphenylpyruvic acid, thus:

$$C_6H_4(OH) \cdot CH_2 \cdot CH(NH_2) \cdot COOH \rightarrow C_6H_4(OH)\,CH_2 \cdot CO \cdot COOH + NH_3$$

p-hydroxy phenylpyruvic acid,

and oxidases which oxidise this acid to melanin. It has been shown however, by Raper and Wormall (60), that tyrosinase does not yield ammonia when it attacks tyrosine, and further that it does not oxidise p-hydroxyphenylpyruvic acid. Another suggestion, put forward by Haehn (34) in 1921, was that tyrosinase is a mixture of an enzyme α-tyrosinase and a co-enzyme which can be separated by ultrafiltration. The co-enzyme consists of inorganic salts and can be replaced by salts of calcium, zinc, cadmium and other metals. The α-tyrosinase is concerned in the splitting up of the tyrosine molecule, and in the building up of the red substance is assisted by the inorganic salts. The red substance he considered to be a very finely dis-

persed colloid which becomes coagulated to give the coarsely disperse phase, melanin. It has been shown both by Wyss (82), and by Raper and Wormall (59), that Haehn's results were probably due to lack of control of pH when testing for the activity of his α-tyrosinase preparations. Raper and Wormall, using potato tyrosinase and working with buffered solutions, have shown that the enzyme is most active between pH 6 and 8, and that outside these limits the activity is small, ceasing finally at pH 5 and 10. In these experiments the substrate used was tyrosine. It was also found that although at pH 6 the tyrosine is quickly converted into a red substance, the subsequent darkening and production of melanin are very slow as compared with the changes at pH 8. This indicates that the first action of the enzyme on tyrosine is the production of the red pigment, and that the later changes occur more readily in an alkaline than an acid medium. It was also discovered that the red substance decolorises on boiling, and that the same change takes place at the ordinary temperature if further oxidation is prevented by keeping the red solution in a neutral atmosphere. By allowing the enzyme to act on tyrosine at pH 6 and then, when the red colour has fully developed, removing the enzyme by means of colloidal ferric hydroxide, a solution of the red pigment was obtained which enabled its properties to be examined. By the use of an enzyme prepared from mealworms (Tenebrio molitor), a solution of the red substance free from the enzyme may also be obtained by acidification and filtration after the red colour is well developed. Raper and Speakman (58) using such a preparation showed that the red substance becomes colourless much more rapidly at pH 8 than at pH 6. When prepared at pH 6 the resulting colourless solution if exposed to air darkens slowly and ultimately deposits melanin. In slightly alkaline solution, however, the darkening is very rapid, and it appears that when the red substance decolorises, it gives rise to a product or products that oxidise easily in the air, and especially so in alkaline solution. These and other experiments led Raper and Wormall to the conclusion that melanin formation from tyrosine takes place in three distinct stages. The first stage is the oxidation of the tyrosine to the red pigment; the second is the decolorisation of the red pigment, and the third is the oxidation of the colourless substance produced in this reaction to melanin. The enzyme is essential only for the first stage, because the second and third stages can take place in its absence. The second stage was regarded as probably an intramolecular change, and not a reduction process brought about by a hydrolytic deamination as suggested by Bach, and later work has shown this view to be the correct one.

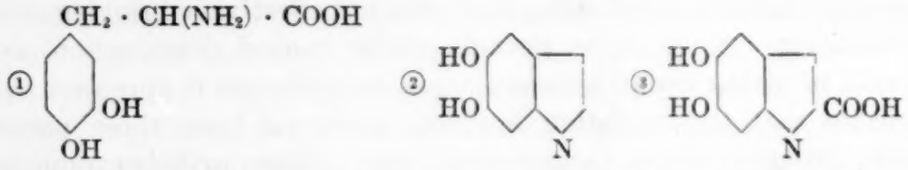
In order to determine the chemical changes which are involved in these reactions, a study was next made of the supposed deaminising action of the enzyme on amino acids. The view that tyrosinase has such an action was first put forward, as stated above, by Chodat and Schweizer and was accepted by Bach, who made this action the basis of a series of reactions by which melanin was supposed to be produced. Folmpmers (28) also isolated benzaldehyde as its p-nitrophenylhydrazone from the products of the action of tyrosinase on phenylaminoacetic acid. Happold and Raper (37), however, were unable to detect either aldehyde or ammonia formation when potato tyrosinase acts on glycine, alanine or phenylaminoacetic acid. It was then observed that successful demonstration of ammonia formation by Chodat and Schweizer had been possible only when p-cresol was present along with the amino acid. These investigators added p-cresol to prevent a supposed interaction of the ammonia and aldehyde liberated during the action of the enzyme. Under these conditions Happold and Raper were able to confirm the results of Chodat and Schweizer. Similar results were obtained independently by Robinson and McCance (62). The fact that deamination of amino acids was obtained only in the presence of p-cresol led Happold and Raper to the view that the deamination was not due to a direct action of tyrosinase on the amino acids but was indirect. It was suggested that the enzyme first oxidised the p-cresol, and the oxidation product then attacked the amino acid with liberation of aldehyde and ammonia.

A study of the action of other phenols introduced into the tyrosinase-amino acid system showed that phenol and catechol had the same action as p-cresol but resorcinol and quinol were not effective in bringing about deamination. Since those phenols which caused deamination were capable of giving rise to ortho-quinones on oxidation it appeared to be probable that the oxidation products produced from those phenols which brought about deamination were either orthobenzoquinone (from phenol and catechol) or 3:4-toluquinone (from p-cresol). This view gained support when it was possible to show that orthobenzoquinone added to glycine or alanine under the same conditions of experiment as were used for the enzyme action, brought about deamination, whereas p-benzoquinone had no effect.

The failure to obtain deamination of amino acids with tyrosinase alone made it appear unlikely that tyrosine itself would be deaminated by the enzyme. On the other hand, tyrosine contains a phenolic hydroxyl group, and it seemed possible that this might have some influence in determining the nature of the reactions produced by the

enzyme. Experiments were therefore carried out to see whether ammonia could be detected at any stage of the action of tyrosinase on tyrosine, but none was found. The fact that the melanin produced by the enzyme from tyrosine contains 8.4 per cent N, which is a little greater than the amount (7.73 per cent) in tyrosine itself, suggests also that deamination does not take place as a result of the enzyme action.

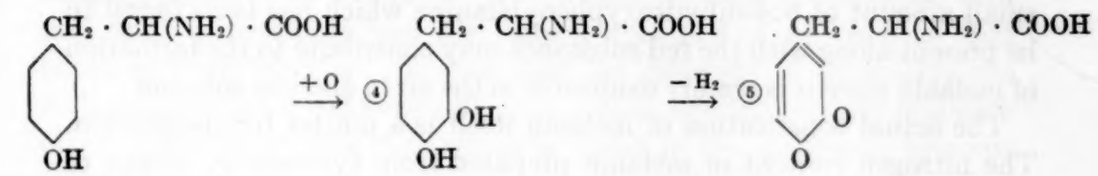
Further progress in elucidating the chemical changes taking place in the oxidation was now made by using a preparation of the enzyme from mealworms (Tenebrio molitor), made by a modification of Gortner's process. This enzyme is completely precipitated by very dilute acids, so that by its use the action on tyrosine can be stopped and the enzyme removed at any stage of the action, by simply acidifying with dilute acetic acid and filtering. Use was also made of the fact that at pH 6.0 the red substance which is produced as the first visible product of the enzyme action is relatively stable. The red substance was therefore obtained by allowing the mealworm tyrosinase to act on a solution of tyrosine at pH 6 until a maximal depth of colour was obtained. Acetic acid was then added and the enzyme removed by filtration, leaving a solution containing unchanged tyrosine along with the products produced from it by the enzyme, one of which is the red substance. It has been shown by Raper (56), (57) that if the solution of the red substance obtained in this way is decolorised either by simply allowing it to stand *in vacuo* or by adding a small amount of sulphurous acid, three substances can be shown to be present, namely, 1, l. 3:4-dihydroxyphenylalanine; 2, 5:6-dihydroxyindole, and 3, 5:6-dihydroxyindole-2-carboxylic acid.



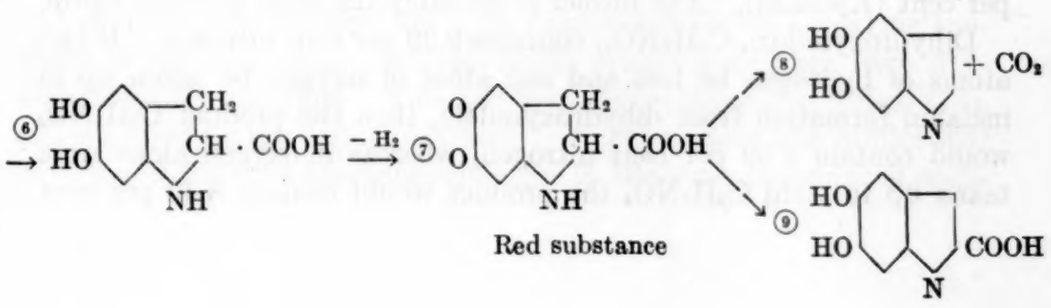
The 3:4-dihydroxyphenylalanine was isolated by first precipitating the decolorised solution, whilst still acid, with lead acetate, and allowing the substances 2 and 3 to oxidise to melanin. This leaves the dihydroxyphenylalanine unchanged, and after filtering, it may be precipitated from the filtrate as the lead compound by making slightly alkaline with ammonia. The lead compound is decomposed with hydrogen sulphide and yields the free amino acid. It was obtained crystalline and in the laevo form, identical with that prepared by Guggenheim (33) from Vicia faba.

The indole derivatives were obtained by allowing the red substance

to decolorise in an atmosphere of hydrogen or in the presence of sulphurous acid. By the first method the free base 2 is obtained principally, and by the second, its 2-carboxylic acid 3. When the red substance has been decolorised by either of these methods, great care has to be exercised to prevent oxidation. The decolorised solution is concentrated in an atmosphere of hydrogen and the products it contains methylated with dimethyl sulphate whilst still under hydrogen. From the alkaline solution resulting after the methylation 5:6-dimethoxyindole was obtained by extracting with ether. On acidifying the solution and again extracting with ether, 5:6-dimethoxyindole-2-carboxylic acid was obtained. Neither of these substances had been synthesised, so their preparation was undertaken by Oxford and Raper (51), and they were found to be identical with the synthetic products. The production of 3:4-dihydroxyphenylalanine by the action of tyrosinase on tyrosine suggests that it is the first oxidation product resulting from the enzyme action. It is known to be readily oxidised by tyrosinase, and much more rapidly than tyrosine itself. It also gives rise to a red substance in the first stages of the action just as tyrosine does. By using the conditions of experiment which enabled 5:6-dimethoxyindole to be isolated from the oxidation products of tyrosine, it has been possible to show that starting with dihydroxyphenylalanine instead of tyrosine, the same indole derivative is obtained. This indicates therefore that 3:4-dihydroxyphenylalanine is the first oxidation product produced from tyrosine. In view of the subsequent formation of the indole derivatives it is suggested that the series of reactions which take place in the tyrosinase-tyrosine reaction preliminary to the formation of melanin are as follows:



Tyrosine



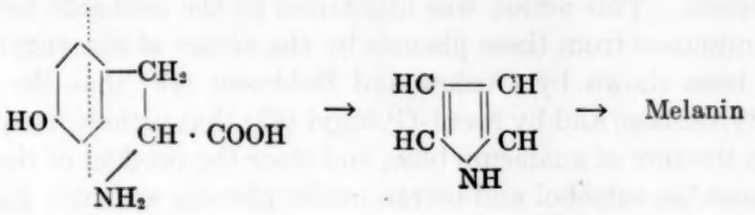
Red substance

The first product is 3:4-dihydroxyphenylalanine, 4, and this is next oxidised to its corresponding quinone, 5. This undergoes intramolecular change analogous to the formation of an anilino-quinone and the N of the side chain attaches itself in the 6 position of the benzene nucleus; the resulting 5:6-dihydroxydihydroindole-2-carboxylic acid, 6, is then oxidised to its corresponding quinone, 7. This is believed to be the red substance which is the first *visible* product of the enzyme action. The enzyme is not necessary for the further stages of the reaction. When the red substance becomes colourless it gives rise to the base, 8, or its carboxylic acid, 9, according to the conditions. If the change is quickened by adding sulphurous acid, which appears to catalyse it, then intramolecular change takes place without loss of carbon dioxide. This is simply an autoreduction whereby 5:6-dihydroxyindole-2-carboxylic acid is produced. If the decolorisation of the red substance is slow, as happens in an inert atmosphere at room temperature, then it also loses carbon dioxide as well as undergoing autoreduction and 5:6-dihydroxyindole is produced. Although 5:6-dihydroxyindole and its 2-carboxylic acid have never been obtained synthetically it is certain that they would be extremely easily oxidised in the air, so that they may rightly be regarded as the precursors of melanin. Under the normal conditions of action of the enzyme, the base is the principal product, so that it would contribute most to melanin formation. The more rapid formation of melanin at pH 8 as compared with pH 6 is explained by the more rapid change of the 5:6 quinone of dihydroindole carboxylic acid into the corresponding indole derivatives on the alkaline side of the neutral point, and by the greater ease of oxidation of these substances at an alkaline pH. It must also be borne in mind that the small amount of 3:4-dihydroxyphenylalanine which has been found to be present along with the red substance may contribute to the formation of melanin since it is readily oxidisable in the air in alkaline solution.

The actual constitution of melanin itself is a matter for speculation. The nitrogen content of melanin prepared from tyrosine by means of tyrosinase by Raper and Wormall is 8.65 per cent (Dumas) and 8.40 per cent (Kjeldahl). The former is probably the more accurate figure.

Dihydroxyindole, $C_8H_7NO_2$, contains 9.39 per cent nitrogen. If two atoms of hydrogen be lost and one atom of oxygen be taken up in melanin formation from dihydroxyindole, then the product $C_8H_5NO_3$ would contain 8.59 per cent nitrogen, whereas if oxygen alone were taken up to yield $C_8H_7NO_3$ the product would contain 8.49 per cent

nitrogen. From these considerations it appears as if in melanin formation from dihydroxyindole one atom of oxygen is taken up for each molecule of the dihydroxyindole oxidised and possibly two atoms of hydrogen are lost. Union of many molecules of the indole most certainly occurs in this process, since melanin is a highly colloidal amorphous substance. It probably also contains quinonoid arrangements because of its deep colour. The analyses of melanin prepared from tyrosine by the tyrosinase reaction do not support the view of Angeli (2) that in melanin formation a pyrrole derivative is first formed and then oxidised to melanin, thus:



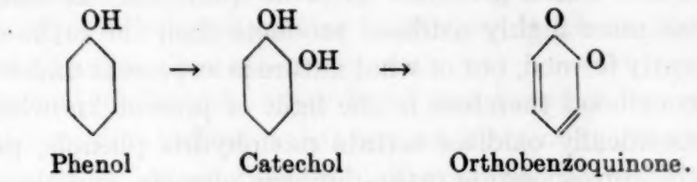
The variable composition of melanins obtained from different natural sources (Bloch and Schaaf, 14) does not necessarily mean that they are not produced by a series of reactions similar to those described above for the melanin produced from tyrosine. It is difficult to isolate melanin free from admixture with other cell components or substances produced from them by the somewhat drastic treatment required for its extraction, and this may account for its high nitrogen content relative to that of melanin from tyrosine. In this connection it may be pointed out that melanin prepared by Brahn (17) from melanotic urine by oxidation with potassium persulphate had the same percentage of nitrogen as that obtained from tyrosine. In this case the difficulty of separating the melanin from other cell constituents is absent, and the product probably more nearly represents that which is found in the melanotic tumours with which melanuria is associated. Other pieces of evidence that melanin is formed in animals by the same changes which account for its formation from tyrosine are; *1*, the existence in the epidermal melanoblasts of the skin of an enzyme which produces melanin from 3:4-dihydroxyphenylalanine. This has been demonstrated by Bloch (13), and is named by him "Dopaoxydase." *2*. Schmalfuss and Müller (64) have found 3:4-dihydroxyphenylalanine in the wing coverings of cock-

chafers (Melolontha melolontha L. and M. hippocastani F.) and Przibram and Schmalfuss have found it in the cocoons of one of the Saturnidae (Samia cecropia L.). These observers regard it as the chromogen which gives rise to melanin in these insects.

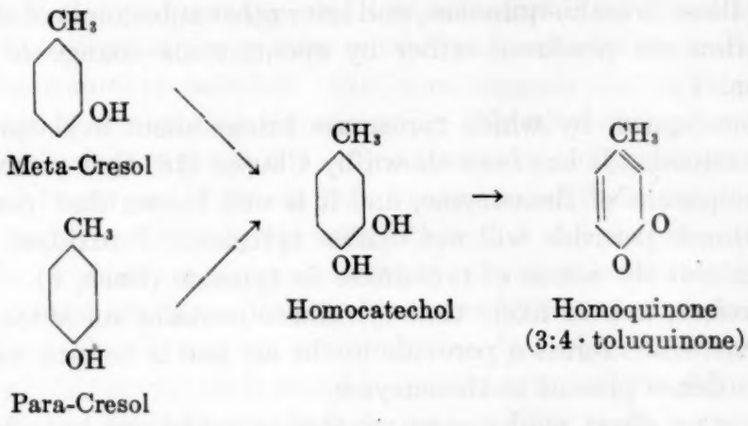
Besides acting on tyrosine and 3:4-dihydroxyphenylalanine, tyrosinase also attacks certain phenols. Chodat and his collaborators have been particularly identified with work in this field and lists of phenols which are oxidised by the enzyme have been published by Chodat (18) and by Wyss (82). It has been pointed out above that Happold and Raper have shown that the products obtained from phenol, p-cresol, and catechol will all cause deamination of an amino acid if it is present in the system. This action was attributed to the probable formation of ortho-quinones from these phenols by the action of the enzyme. It has also been shown by Onslow and Robinson (49) that the system p-cresol-tyrosinase, and by Szent-Györgyi (67) that orthobenzoquinone, both turn tincture of guaiacum blue, and since the product of the action of tyrosinase on catechol and certain other phenols will turn guaiacum blue, it has been suggested by the latter that orthoquinonês are produced by the action of the enzyme on these phenols.

More direct evidence of the production of ortho-quinones has recently been published by Pugh and Raper (55). The ortho-quinones are so unstable that they cannot be isolated directly, but by introducing aniline, which is not attacked by tyrosinase, into the reaction system, it has been possible to isolate the ortho-quinones in the form of their anilino compounds. These separate out during the course of the reaction as red crystalline substances which may be purified by recrystallisation from acetone. Anilinoquinones have been obtained in this way from phenol, catechol, m-cresol, p-cresol and homocatechol. Phenol and catechol give rise to the same anilinoquinone, which has been identified by synthesis from ortho-benzoquinone and aniline. The two cresols and homocatechol give rise to the same dianilinohomoquinonanil, which has also been identified by its synthesis from homoquinone (3:4 toluquinone) and aniline. These experiments leave no doubt that the mode of action of tyrosinase on the monohydric phenols is, first, to introduce a hydroxyl group into the ortho position to the one already present, and secondly, to oxidise the catechol or catechol derivative thus produced to its corresponding ortho-quinone. So that the

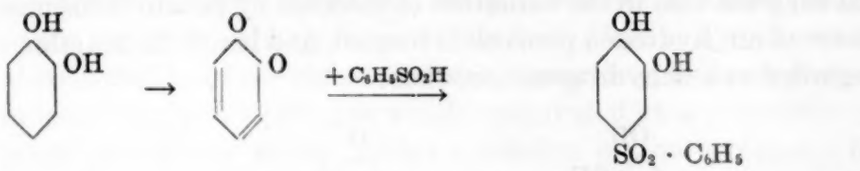
reactions occurring with phenol, m-cresol and p-cresol, may be represented as follows:



Phenol Catechol Orthobenzoquinone.



It has also been shown that m- and p-methoxyhydroxybenzene behave like the corresponding cresols. Another method for the isolation of ortho-quinone derivatives was also tried, namely, the introduction into the reaction system of benzenesulphinic acid. This unites with quinones to produce sulphones (Hinsberg and Himmelschein, 40). It was not successful when tried along with tyrosinase and phenol, but it was when catechol replaced the phenol. In this way ortho-dihydroxydiphenylsulphone was isolated from the reacting system.



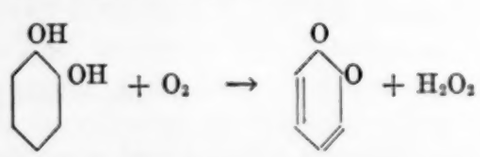
Catechol O · dihydroxydiphenylsulphone

Ortho-quinones are, however, not the end products of the action of the enzyme. If the oxygen uptake is measured when a known amount of p-cresol is oxidised by tyrosinase, it amounts to about 3 atoms per molecule of p-cresol, and 2 atoms when catechol is the substrate (Robinson and McCance, 62). For the formation of the ortho-quinone, in

these two instances 2 and 1 atoms of oxygen are required respectively. These results have been confirmed and extended by Pugh and Raper (55) to other phenols which give rise to ortho-quinones. It would seem therefore that more highly oxidised products than the ortho-quinones are subsequently formed, but of what nature is at present undetermined. It may be concluded therefore in the light of present knowledge that tyrosinase specifically oxidises certain monohydric phenols, producing from them the corresponding ortho-dihydric phenols, and then in turn oxidises these to ortho-quinones, and later other substances of unknown constitution are produced either by spontaneous change or further oxidation.

The mechanism by which tyrosinase brings about oxidation is not yet understood. It has been shown by Chodat (18) that peroxidase is not a component of the enzyme, and it is well known that peroxidase and hydrogen peroxide will not oxidise tyrosine. Peroxidase is even said to inhibit the action of tyrosinase on tyrosine (Bach, 6). It does not, therefore, appear likely that tyrosinase contains an autoxidisable component which forms a peroxide in the air and is in turn activated by a peroxidase present in the enzyme.

There is no direct evidence as yet that in oxidations brought about by tyrosinase, oxygen can be replaced by a suitable hydrogen acceptor. Raper and Wormall showed that no oxidation of tyrosine by the enzyme prepared from potatoes took place *in vacuo* or in an atmosphere of hydrogen even if methylene blue were present. It has also been shown by Onslow and Robinson (50) that a catechol-oxidising enzyme (probably tyrosinase or a polyphenolase) prepared from various sources was not able to oxidise catechol *in vacuo* in the presence of various hydrogen acceptors. These observers however have brought forward evidence which suggests that in the oxidation of catechol by potato tyrosinase, in presence of air, hydrogen peroxide is formed, and hence the reaction may be regarded as a dehydrogenation, thus:



Reactions of this type leading to the production of hydrogen peroxide in the presence of molecular oxygen are usually taken as an indication that in the oxidation process the hydrogen becomes activated by the enzyme. But if this is so it is not yet satisfactorily explained why other

hydrogen acceptors, e.g., methylene blue, cannot replace oxygen. It appears to the author much more probable that tyrosinase acts by activating oxygen which is thereby able to bring about the dehydrogenation of catechol and similar substances much as peroxidase and hydrogen peroxide do.

McCance (46) on the other hand has brought forward evidence which he considers indicates that oxygen is not obligatory in oxidations by tyrosinase. Certain mono- and dihydric phenols will reduce methylene blue if glycine is present, and this reduction is catalysed by tyrosinase. The effect of the enzyme is more marked with m- and p- cresol than with quinol or catechol. McCance suggests that in this reaction tyrosinase is accelerating the anaerobic oxidation of a phenol, and is acting by hydrogen activation. But tyrosinase, in agreement with the results of Raper and Wormall (59), was not found to catalyse the reduction of methylene blue by tyrosine although it is a phenolic amino acid and may be considered as containing in its molecule the equivalent of a phenol and glycine. Hence, McCance thinks that this is due to methylene blue being an inappropriate hydrogen acceptor for the reaction. To prove this it would be necessary to find the right hydrogen acceptor, but this has not yet been done. Until more is known about the reaction which occurs when phenols and glycine react and reduce methylene blue, the significance of McCance's results for the interpretation of the action of tyrosinase on tyrosine and the phenols cannot be determined.

Onslow and Robinson (49) have suggested that for the action of tyrosinase on tyrosine either a trace of an ortho-dihydroxy-compound, possibly 3:4 dihydroxyphenylalanine, is an essential impurity in the tyrosine or alternatively, the enzyme contains traces of active oxygen which produce such a dihydroxy compound when it comes in contact with the tyrosine. On the basis of the Bach-Chodat hypothesis of the dual constitution of the direct oxidases, the ortho-dihydroxy compound is supposed to form a peroxide which is activated by a peroxidase in the enzyme and brings about further oxidation of the tyrosine. It has been shown by Raper (56) that traces of 3:4-dihydroxyphenylalanine do accelerate the oxidation of tyrosine in the early stages of the enzyme action, but there is no evidence as yet that the mechanism is that suggested by Onslow and Robinson.

Whether tyrosinase has any function in animals and plants besides the formation of melanin is not known. Its presence has not yet been established even in all cells which produce melanin, e.g., the melano-

blasts of the epidermis, though there is no doubt that it occurs in considerable amounts in lower animals which actively produce melanin. It is thus not clear that tyrosinase is necessarily concerned in melanin production in the higher animals; but the investigation of this subject has been so slight that it is not possible in the present state of our knowledge to arrive at any conclusions on the subject. On the other hand, Szent-Györgyi (67) has attempted to show that the enzyme has some importance in the respiratory processes of plants. From the potato, Szent-Györgyi has obtained a substance which he calls "tyrin." This is capable of "oxidation" to a red substance "oxytyrin" by a tyrosinase-catechol system or by ortho-quinone. The red substance becomes colourless on reduction, and is regarded as a hydrogen acceptor which may function in plant respiration, thus:

Molecular oxygen	+ tyrosinase	+ catechol →	ortho-quinone
Ortho-quinone	+ leuco-tyrin	→ catechol +	oxytyrin
Oxytyrin	+ "active" hydrogen →	leuco-tyrin	

In this scheme tyrosinase by virtue of its action on catechol is able to oxidise the tyrin, which by itself is not oxidised by molecular oxygen. The oxytyrin thus produced acts as a hydrogen acceptor for activated hydrogen and is reduced to leucotyrin again. It has however been shown by Platt and Wormall that tyrin owes all its so-called "oxidative" properties to the presence in it of free or combined amino acids and these can be imitated by a mixture of amino acids. Since ortho-quinone deaminates amino acids and combines with them irreversibly to form a series of pigments (Chodat, 18), tyrin cannot be regarded as having the functional value assigned to it by Szent-Györgyi.

Because of its action on monohydric phenols to produce orthodihydric phenols, tyrosinase may be of importance in the production in nature of catechol compounds, and for similar reasons it may be concerned in the production of adrenalin from tyrosine. The apparent absence of tyrosinase from the higher animals is an obstacle to the acceptance of this last suggestion, but further investigation is required.

Laccase and polyphenolases. There is an ill-defined group of aerobic oxidases which do not oxidise tyrosine, and are therefore different from tyrosinase, and which do not require the addition of hydrogen peroxide for their characteristic oxidation reactions. They are thus not simple peroxidases. In air they turn guaiacum blue and have therefore been called "direct oxidases" to distinguish them from peroxidases which require the addition of a peroxide before they will bring about this reac-

tion. The first example of this group to be identified was laccase. It was discovered in 1883 by Yoshida (83), who isolated it from the Japanese lac tree, Rhus vernicifera. In 1894 Bertrand (8) commenced an investigation of this enzyme, and in succeeding years was able to show that it was present in many plants and fungi. The guaiacum reaction ("direct oxidase" reaction) was largely used by him for its detection. In addition, its action on a series of phenolic substances was demonstrated and it was found not to oxidise tyrosine. It was thus definitely distinguished from tyrosinase. Bertrand's preparation of the enzyme contained manganese, and its oxidising power was found to be proportional to the manganese present. For instance, three specimens of laccase containing 0.159, 0.126 and 0.098 per cent of manganese, when acting upon a solution of quinol for the same time used up 19.1, 15.5 and 10.6 cc. of oxygen respectively. Laccases of varying activity were also prepared by Bertrand (10) from other sources. From Lucerne, (Medicago sativa), a preparation was obtained which was only slightly active and contained only minute amounts of manganese. On addition of a trace of manganese the activity was considerably increased. The oxygen absorbed in 6 hours by 50 cc. of a 1 per cent solution of quinol when the Medicago laccase alone was added was 0.2 cc., and when 1 mgm. manganese sulphate alone was added was 0.3 cc., but with the laccase and manganese salt together was 6.3 cc.,—a twentyfold increase. Bertrand (11) also showed that manganese salts, particularly those of organic acids, would accelerate the aerobic oxidation of quinol and he concluded from these experiments that manganese was the active agent in laccase. The manganese was supposed to be combined with some specially appropriate acid radicle, probably a protein. The salt thus formed was considered to undergo hydrolytic dissociation yielding MnO which reacting with molecular oxygen would give rise to MnO_2 and "active" oxygen, the latter oxidising the substrate. The MnO_2 reacting with the acid produced by hydrolytic dissociation yields the manganese salt again and free oxygen which will also bring about oxidation. It is clear that Bertrand could not conceive of an active laccase which did not contain manganese.

Concerning the substrates on which the enzyme acts, he stated in 1898, "The direct oxidation of monophenols and monoamines is not, or almost not, influenced by laccase. It acts best on aromatic substances containing at least two OH or NH_2 groups in the nucleus and in which these groups are in the *ortho* or *para* position to each other." It must be remembered in considering the significance of Bertrand's results

that at the time they were obtained peroxidase and catalase had not been distinguished, so that he was unaware of the existence of peroxidases as separate enzymes. It is possible that the manganese compounds in some, at least, of his preparations of the enzyme acted by producing hydrogen peroxide which in the presence of peroxidase would cause oxidation of the phenolic substrate.

In 1902, Bach and Chodat (3) showed that peroxides might be formed in plant juices on exposure to air, and they succeeded in obtaining a peroxide from the juice of Lathraea squamaria by aeration in the presence of baryta. If the juice had been kept for so long a time that it no longer gave the direct guaiacum reaction then this treatment did not yield a peroxide. They were thus able to relate the direct guaiacum reaction in plants to the formation of peroxides in air. A year later the same observers (4) showed that by fractional precipitation with alcohol of the juice of Lactarius vellereus, which gives a strong direct guaiacum reaction, it was possible to obtain two fractions, one of which oxidised guaiacum only faintly, the other not at all. Together they gave a strong guaiacum reaction. The former could be activated by peroxidase from different sources, the latter itself activated hydrogen peroxide and was obviously rich in peroxidase. The former they called an "oxygenase," and they regarded it as an oxygen carrier which forms a peroxide on exposure to air. This peroxide could then be activated by a peroxidase. Bach and Chodat pointed out also that Bertrand's results with laccase did not disagree with their own, since the manganese in laccase could be regarded as functioning in a manner analogous to that of oxygenase in lactarius, i.e., as an oxygen carrier. They also pointed out that manganese could not be regarded as an essential constituent of the direct oxidases, because peroxidase prepared from horseradish contains manganese, yet does not give the direct guaiacum reaction. A further study of the nature of laccase was begun in 1908 by Euler and Bolin (26). They showed that the laccase present in Medicago sativa was entirely different in composition from that in Rhus succedanea and vernicifera, as it contained practically no manganese, was thermostable, and did not oxidise guaiacum directly. It was found to consist of a mixture of calcium salts of organic acids which catalysed the oxidation of polyphenols in the presence of traces of manganese salts. They imitated its action successfully, and apparently exactly, with a mixture of the calcium salts of glycollic, malic and mesoxalic acids. These experiments supported those of Bach and Cho-

dat in showing that direct oxidases did not necessarily have the constitution assigned to them by Bertrand.

Bach and Chodat's theory of the dual nature of the direct oxidases has generally remained unassailed up to the present, but facts which have accumulated in recent years indicate that it should be revised. A modification of the theory was suggested by M. W. Onslow (48) in 1920 as a result of her investigation of the cause of darkening of the juices of plants, fruits, etc., on exposure to air. Such material was found to give the direct oxidase reaction and on treatment with alcohol it was possible to separate from it an alcohol-insoluble enzyme, and an alcohol-soluble substrate, which separately had no action on guaiacum, but together gave a marked direct oxidase reaction. The alcohol-soluble extract was found to give a green colour with ferric chloride and was therefore assumed to contain a catechol derivative. It was found that it could be replaced by catechol and several catechol derivatives. Onslow's interpretation of these phenomena was that the oxygenase of Bach and Chodat was really a mixture of an enzyme and a catechol derivative which reacting together in presence of air gave rise to a peroxide, this in turn being acted on by peroxidase to give "active" oxygen which turns guaiacum blue. It was suggested by Onslow that the term oxygenase should be retained for the enzyme in this catechol-enzyme combination since it possessed the usual properties of enzymes, whereas this was not always obviously true of Bach and Chodat's oxygenase. Onslow did not obtain the catechol-oxidising enzyme free from peroxidase, so that the importance of the latter in her scheme for the direct oxidase reaction of plant juices remained uncertain. It was shown, however, in 1925 by Szent-Györgyi (67) that ortho-quinone will turn guaiacum blue, and it was suggested by him that ortho-quinones are produced by the action of plant oxidases on catechol and certain other phenols. That such a supposition is correct for tyrosinase has been shown recently by Pugh and Raper, and that it also applies to another plant oxidase follows from the work of Wieland and Fischer (75) on an oxidising enzyme which they isolated from Lactarius vellereus and which oxidises catechol and quinol to the corresponding quinones.

These discoveries render almost certain the identity of the oxygenase of Onslow with either tyrosinase or a polyphenolase of the laccase type. Both these enzymes oxidise catechol in the air to produce ortho-quinone and if guaiacum is added it will be turned blue. It does not therefore seem necessary to retain the term oxygenase as applying to a new enzyme in the sense used by Onslow, unless it can be shown that both

tyrosinase and an enzyme of the laccase type are absent from plants that give the oxygenase-catechol reaction. That this is possible seems very unlikely in view of the wide occurrence of these enzymes.

The most recent work on laccase or an oxidase of the laccase type is that by Wieland and Fischer referred to above. The primary object of this investigation was to find out whether plant oxidases act by dehydrogenation. The source of the enzyme was the juice of the fungus Lactarius vellereus which was purified by fractional precipitation with alcohol. The purest samples obtained contained little or no catalase and apparently no peroxidase. In the ash neither iron nor manganese could be detected. The enzyme withstood heating in aqueous solution at 100° for 20 minutes with little depreciation of its activity, but it was different from the mixture of calcium salts of organic acids isolated from Medicago sativa by Euler and Bolin which had some of the properties of Bertrand's laccase and was, of course, thermostable.

Using pyrogallol or quinol as substrate, it was found by Wieland and Fischer that although their catalyst brought about oxidation in the air it could not do so in a neutral atmosphere in the presence of any of the following hydrogen acceptors: dinitrobenzene, methylene blue, sodium nitrite, alloxan, cystine, dithioglycollic acid, hydrogen peroxide, diethyl-peroxide. The enzyme—if this term may be applied to a thermostable substance—appears therefore to belong to the class of aerobic oxidases as defined earlier in this review. In the presence of a trace of hydrocyanic acid to inhibit the action of the traces of catalase it contained, the enzyme oxidised quinol with the production of equal amounts of p-benzoquinone and hydrogen peroxide, thus:

$$C_6H_4(OH)_2 + O_2 = C_6H_4O_2 + H_2O_2$$

Wieland and Fischer therefore conclude that it acts by activation of hydrogen and hence gives rise to dehydrogenation, and not by activation of oxygen. They suggest that the enzyme, quinol and molecular oxygen, combine to form an unstable compound which then breaks down yielding the quinone and hydrogen peroxide.

It should be pointed out however that the fact that hydrogen peroxide is produced indicates merely the acceleration of a normal process since catechol on autoxidation in the air produces hydrogen peroxide (Onslow and Robinson, 50) and probably quinol does the same. The hydrogen of the phenolic hydroxy groups in these substances may be considered as already activated because they have well-marked reducing properties. It is therefore just as reasonable to suppose that the catalysis of this

oxidation process occurs by activation of oxygen as by greater activation of hydrogen. The fact that common hydrogen acceptors are not reduced by quinol in presence of the catalyst would suggest that the more likely explanation of the action lies in oxygen activation.

The fact that hydrogen peroxide is produced in this reaction, in presence of excess of the substrate, excludes the presence of peroxidase. Further evidence of the absence of peroxidase is given by the inability of the enzyme to attack quinol in an atmosphere of nitrogen when hydrogen peroxide is present. In addition to quinol and pyrogallol, the enzyme produced hydrogen peroxide when acting on catechol, guaiacol and anthrahydroquinone. Whether it turned guaiacum blue directly is not stated, nor whether it oxidised tyrosine. The latter is unlikely, as the thermostability of the enzyme and its action on guaiacol rule out identity with tyrosinase. But from the reactions which Wieland and Fischer describe it is evident that this enzyme may be classed as a direct oxidase. Since it does not give peroxidase reactions there is no evidence that it has the dual nature ascribed to direct oxidases by Bach and Chodat. Nor is it a substance which by autoxidation forms a peroxide which is subsequently hydrolysed to yield hydrogen peroxide, so that it does not possess the properties of an oxygenase. The absence of manganese shows that it does not depend for its action on manganese ions like the laccase of Euler and Bolin or Bertrand's laccase from Rhus vernicifera. But the last-named enzyme certainly requires re-investigation from this point of view, since it is uncertain whether Bertrand's original laccase contained peroxidase, or whether manganese was absolutely essential for its action.

A survey of the known facts regarding aerobic oxidases shows that the direct guaiacum reaction can no longer be taken as an indication of the presence of any particular enzyme or combination of enzymes. All of the following oxidation systems give this reaction, and the components may and do occur together in many plant tissues:

1. Peroxidase together with an autoxidisable substance which produces a peroxide in air. This is the oxygenase-peroxidase combination of Bach and Chodat. There are many substances that could function as peroxide formers, e.g., thiol compounds, terpenes, unsaturated fats or fatty acids, lipines, possibly manganese salts of organic acids (Bertrand). Gallagher (29) for example, has found a system of this type in the potato, the autoxidisable substance being a lipine or mixture of lipines.

2. Peroxidase together with an oxido-reductase (dehydrogenase) and

its substrate. The oxido-reductases act by activating hydrogen, and when oxygen acts as an acceptor for this, hydrogen peroxide is produced. This hydrogen peroxide in the presence of peroxidase will serve to bring about a secondary oxidation if an appropriate substrate is present. An example of a system of this kind is that investigated by Thurlow (69) in her study of the xanthine oxidase of milk. This oxidase when oxidising xanthine or hypoxanthine in presence of air produces hydrogen peroxide. If peroxidase is present also, then a secondary oxidation of a substrate which is oxidised by the peroxidase-peroxide system can be brought about concurrently.

3. Peroxidase together with a polyphenolase in presence of a polyphenol, e.g., quinol. In a system of this kind the polyphenolase may be one of the type investigated by Euler and Bolin (26), which requires the presence of traces of manganese in order that the quinol may be oxidised, or it may be one such as Wieland and Fischer isolated from Lactarius vellereus. Either will produce hydrogen peroxide from the polyphenol, and in the presence of peroxidase would give rise to a secondary oxidation.

4. A polyphenolase together with catechol or one of its derivatives. This gives rise to an ortho-quinone which will oxidise guaiacum directly. M. W. Onslow's "oxygenase"—catechol system comes into this group as well as the next one.

5. Tyrosinase together with one of its substrates (tyrosine, 3:4-dihydroxyphenylalanine and pyrogallol excepted). It has been shown by Szent-Györgyi (67) and by Pugh and Raper (55) that orthoquinones are produced in systems of this kind, and they will therefore oxidise guaiacum directly.

From a consideration of the composition of these various systems it is clear that a positive direct oxidase reaction with guaiacum does not show the presence of any particular oxidising enzyme or system, although it does indicate that peroxidase, tyrosinase or a polyphenolase may be present.

This reaction will not, however, distinguish between them. Nor does it necessarily indicate the presence of a dual oxygenase-peroxidase system of the Bach-Chodat type. There is no simple test which distinguishes these five systems; but a rough division may be achieved by the use of tyrosine as a substrate, since absence of tyrosinase rules out system 5 in the above series.

The identification of peroxidase and polyphenolases when present together is difficult since they both give the same reactions with various

substrates. So far the only method which has given any measure of success has been to separate them by fractional precipitation with alcohol, this method being the one used by Wieland and Fischer in preparing a polyphenolase from the juice of Lacterius vellereus. The peroxidase is more soluble and comes down in the later fractions as a rule. Whether this method would answer equally well with other plant materials is not certain.

DOPA OXIDASE AND INDOPHENOL OXIDASE. These are two enzymes or substances which appear to be enzyme-like in their action which have a different distribution from those already considered, since both are regularly met with in higher animals. They appear to be somewhat analagous to the polyphenolases and may be conveniently considered along with them.

Dopa oxidase has been so named by Bloch (13), because it selectively oxidises 3:4-dihydroxyphenylalanine ("dopa") to melanin. It is found in the melanoblasts of the epidermis, and its presence there may be demonstrated by treatment of sections of skin that have been fixed in formaldehyde in the usual way, from a few hours up to 3 days, with a 0.1 per cent solution of pure 3:4-dihydroxyphenylalanine, buffered to pH 7.3 to 7.4. After 24 hours in this solution at room temperature the cells which contain the enzyme are deeply stained with melanin. It is only the pigment-forming cells (melanoblasts) of the epidermis which give the reaction. The melanophores are unaltered. Bloch and Schaaf (14) and Albl (1) have succeeded in extracting the enzyme from the skin of newborn rabbits, using a technique similar to that used by H. Onslow (47) in which he demonstrated the presence of an enzyme which darkened tyrosine solutions in the presence of hydrogen peroxide. According to Bloch and Schaaf the enzyme is destroyed by heating the extract to 100°C. and its action is inhibited by M/500 KCN or M/2000 H_2S. No enzyme was obtained from the skin of albino rabbits. Dopa oxidase is very specific in its action. It does not give the typical reaction with any of the following substances: *Monophenolic substances;* tyrosine, p-hydroxyphenylpyruvic acid, p-hydroxyphenylethylamine; *Catechol derivatives;* catechol, protocatechuic acid, p-oxy.m.methoxy-phenylalanine, caffeic acid, 3:4-dihydroxyphenylaminoacetic acid, adrenaline, glycyl-l-3:4 dihydroxyphenylalanine; *Other polyphenolic substances;* quinol, homogentisic acid, 2:5-dihydroxyphenylalanine, pyrogallol, 2:3:4-trihydroxyphenylalanine, 3:4:5- trihydroxyphenyla-lanine. *Other cyclic substances;* pyrrole, tryptophane.

It seems very probable that the processes by which melanin is formed

from tyrosine, which were described above in the section dealing with tyrosinase, are the same as those which result in its production from 3:4-dihydroxyphenylalanine by dopa oxidase. It has already been shown by Raper that 3:4-dihydroxyphenylalanine is the first product in this reaction when tyrosine is the substrate, and this gives sufficient ground for the belief that the subsequent processes which result finally in melanin formation will be the same in both instances. The extreme specificity of this enzyme and the difficulty of extracting it from cells that contain it suggest that its specificity may be due to selective combination of the substrate with some substance or surface inside the melanoblasts. Otherwise it is difficult to understand why other polyphenols are not oxidised by it. It is not a tyrosinase since it does not oxidise tyrosine in air. The extract prepared from skin by H. Onslow, which was referred to above, did oxidise tyrosine, but only when hydrogen peroxide was added. This would apparently exclude tyrosinase also, since this enzyme should oxidise tyrosine in air without any addition. It is, however, possible that in making the extracts some substance is included which inhibits the tyrosinase reaction and that this inhibitory influence is removed by hydrogen peroxide. But there are other inhibitors also as shown by H. Onslow, since extracts of skin from white areas of dominant white rabbits inhibited the oxidation of tyrosine by extracts made from pigmented areas, even when hydrogen peroxide was present. The question of the presence of tyrosinase in the melanoblasts is certainly worth further investigation in view of the suggestion of Bloch that 3:4-hydroxyphenylalanine is normally produced in melanoblasts, and also because the most likely method of its production would appear to be the oxidation of tyrosine by tyrosinase.

Indophenol oxidase. The indophenol reaction was discovered in 1885 by Ehrlich (25), who showed that the tissues of animals injected with p-phenylenediamine and α-naphthol turned blue, due to the formation of indophenol. The reaction with animal tissues *in vitro* was studied by Röhmann and Spitzer (63) ten years later, using a dilute alkaline solution of the above two reagents as substrate. This solution turns blue very slowly in air but much more rapidly in contact with many animal tissues or with extracts made from them. The reaction was considered by Röhmann and Spitzer to be due to a single oxidising enzyme with a wide distribution in the body. Dry preparations which retained their activity for a year were obtained by precipitating aqueous extracts of various tissues with alcohol which precipitated the active constituent. Pohl (54) showed in 1896 that plant tissues

also give the indophenol reaction and in some cases very strongly. The earlier work on this oxidase as well as others is well summarised by Kastle (42), and need not be dealt with here. An improvement in the investigation of the quantitative distribution of the enzyme was made in 1912 by Batelli and Stern (7), who estimated its amount by the increase in oxygen uptake which was produced when p-phenylenediamine was added to various tissues. They showed that this was more exact than the the intensity of colour production with Röhmann and Spitzer's reagent for several reasons. First, α-naphthol inhibits oxidation processes. Secondly, not all the oxidation products of p-phenylenediamine react with α-naphthol to give indophenol blue, and the more vigorous the oxidation the less blue is produced. It may therefore happen, paradoxically, that with the more rapid oxidation obtained at body temperature, a smaller development of blue colour may occur than in the cold, when oxidation is slower. Thirdly, indophenol blue is decolorised by the reducing action of some tissues, particularly liver and kidney, and its production may be therefore missed.

Batelli and Stern showed that all tissues of higher animals oxidise p-phenylenediamine. The most active are brain, blood corpuscles, heart, red muscle, kidney and liver. Pancreas, spleen and lung are less active. Pale muscle is not so active as red. Washed tissues also oxidise p-phenylenediamine energetically, but after heating to 60°C. for ten minutes or treating with alcohol or acetone, the property is lost. Hemoglobin oxidises p-phenylenediamine in the presence of hydrogen peroxide, and Batelli and Stern attributed the oxidative activity of tissue extracts in the presence of hydrogen peroxide, to the presence in them of hemoglobin which acts like a peroxidase in this reaction. The action of washed tissues is, however, probably not due to a peroxidase, since tissues treated with alcohol or acetone, which do not destroy peroxidase, lose their power to oxidise p-phenylenediamine directly, and do not recover it in the presence of hydrogen peroxide. From these and other observations, Batelli and Stern classified the substance in washed tissues responsible for the oxidation of p-phenylenediamine as an oxidase. In the scheme adopted in the present review it would be associated with the aerobic oxidases. They were not satisfied that it was identical with the succinoxidase in brain tissue, and regarded it as a matter of doubt whether in other tissues two separate enzymes existed for the oxidation of p-phenylenediamine and succinic acid respectively. This work, therefore, did not definitely identify the indophenol oxidase with any particular tissue oxidation process of physiological importance.

An attempt to do this was made in 1925 by Szent-Györgyi (66), who attributed to indophenol oxidase in animals a function similar to that suggested by Palladin (52) for plant oxidases. According to Palladin's views, plant oxidases do not act directly on the substance which is to be irreversibly oxidised, but rather on phenolic substances in the plant tissues, converting them into quinones which have a higher oxidising power than the original phenol, or as we should say now, into acceptors of activated hydrogen. The quinones are then again reduced to phenols by the enzyme. The phenolic substances together with the oxidase function in this way as oxygen carriers.

The scheme is represented thus:

$$\text{Combustible substance} + \text{quinone} \xrightarrow{\text{dehydrogenase}} \text{quinol} + \text{oxidation products of the combustible substance}$$

$$\text{Quinol} + \text{oxygen} \xrightarrow{\text{oxidase}} \text{quinone} + \text{water}$$

The experiments of Szent-Györgyi were intended to demonstrate that the indophenol oxidase and p-phenylenediamine could act in a similar manner in the oxidation of lactic acid by muscle. In this instance the di-imine, $C_6H_4=(NH)_2$, is formed and not the quinone, and this in turn is reduced again to the diamine. Szent-Györgyi took washed muscle which had been ground up with powdered glass, suspended it in phosphate solution and added p-phenylenediamine. This was quickly oxidised to the di-imine, which has a blue colour. On evacuating in a Thunberg tube the di-imine was not decolorised because no hydrogen donator was present, but on adding sodium succinate or lactate, which serve as hydrogen donators, reduction of the di-imine took place. It is well known that in presence of oxygen, succinic acid is oxidised by washed muscle and that lactic acid is not. Szent-Györgyi attributes this difference to the spatial separation, in the insoluble muscle substance, of the dehydrogenase which activates the hydrogen of lactic acid and the oxygen activator which converts molecular oxygen into a hydrogen acceptor. The similar activators which bring about the oxidation of succinic acid are supposed to be in closer spatial relationship. The rôle of p-phenylenediamine he assumes to be that of an oxygen carrier which thus links up the oxygen activation centre with the lactic acid dehydrogenase. Evidence is brought forward by him to show that in the presence of air, washed muscle will oxidise lactic acid

if p-phenylenediamine is added. The actual experiments show that the system: washed muscle-oxygen-p-phenylenediamine, after allowing time for the diamine to be oxidised, subsequently takes up more oxygen if sodium lactate be added, thus affording an indication that the lactic acid is being oxidised. According to Szent-Györgyi in living muscle a co-enzyme is present which plays the part of p-phenylenediamine in the above system. Attempts to demonstrate the presence of this co-ferment failed because of the difficulty of separating it from the hydrogen donators which fresh muscle contains. The idea is no doubt an attractive one, but in 1926 Handovsky (36) published a series of experiments which led to conclusions quite different from those of Szent-Györgyi. Handovsky was able to confirm the results of Szent-Györgyi only when the washed muscle was ground up with glass powder. When washed muscle without the glass powder was used there was no evidence that the oxidation of lactic acid was increased. Handovsky attributes Szent-Györgyi's positive results to the adsorption of lactic acid by the glass powder and its subsequent displacement by the p-phenylenediamine thus allowing it to be oxidised. Results conforming with this explanation were also obtained by substituting charcoal for the glass powder. Handovsky showed also that charcoal was only slightly inferior to washed muscle as a catalyst in the oxidation of p-phenylenediamine. In other words, charcoal can act as an indophenol oxidase. He concludes that oxidation of the diamine is accelerated on surfaces, and is largely a manifestation of a physical process, i.e., adsorption, rather than a chemical activation. With quinol this is not so, since washed muscle oxidises this substance much more effectively than charcoal.

There are other observations, however, which suggest that Handovsky's conclusions cannot be accepted as an adequate explanation of the nature of the catalysis of the oxidation of p-phenylenediamine by tissues. Charcoal frequently contains traces of iron, and Warburg (71) has brought forward experimental evidence to show that the iron, although small in amount, plays a very important part in the catalysis of oxidations on charcoal surfaces. He has also shown (72) that the respiration of yeast is inhibited by carbon monoxide and that this inhibition is prevented by light. The influence of light on the combination of carbon monoxide with complex, organic, iron compounds, e.g., hemoglobin, suggests, as indicated by Warburg, that the inhibibition of the respiration of yeast is due to the combination of carbon monoxide with some complex iron-containing component of the yeast which plays

an important part in its respiratory activities. J. B. S. Haldane (35) has also shown that carbon monoxide inhibits the respiration of moths and cress plants when its amount relative to oxygen in the atmosphere to which these are exposed exceeds a certain figure. Finally, Keilin (44) has found that carbon monoxide and cyanides inhibit the indophenol oxidases both of yeast and mammalian muscle. These experiments indicate that the oxidation of p-phenylenediamine, although it may be preceded by surface adsorption, is not due solely to this but to the presence in almost all living tissues which use oxygen of one, or as Haldane suggests, possibly several iron-containing compounds which are oxygen activators. Their normal function cannot yet be definitely stated. There is no evidence that it is solely to oxidise polyphenols. Although these substances occur in plants, and may be of importance in respiration there, they have not been recognised in animal tissues to an extent which would make them seem to be of much general significance. But the wide distribution of the indophenol oxidases does certainly indicate that they are of fundamental importance in tissue respiration. They are not, however, always essential in the oxidation processes of animal tissues since Dixon (23) has recently shown that the aerobic oxidation of acetaldehyde or hypoxanthine by the milk oxidase is not inhibited by carbon monoxide, and Dixon and Thurlow (24) have previously shown that this oxidase is not affected by cyanide. That cyanide and carbon monoxide do not necessarily inactivate the same substance has also been shown by Dixon (23), who found that the succinoxidase of muscle was not inhibited by carbon monoxide, although it is strongly inhibited by cyanides (Fleisch, 27).

THE FUNCTION OF AEROBIC OXIDASES. It appears from the evidence which has been described above that certain oxidases act only in the presence of air and not in the presence of the usual hydrogen acceptors. For these the name aerobic oxidases has been suggested, as opposed to those oxidases which will act in the absence of oxygen provided a suitable hydrogen acceptor is present, and which may be called anaerobic oxidases. It seems probable that this difference in properties connotes an essential difference in their mode of action. It is generally accepted that the anaerobic oxidases activate hydrogen in the substrate on which they act. In other words, they may convert substances which are not commonly regarded as reducing agents into such. The oxidation of succinic acid is a good illustration of this. Under normal conditions *in vitro* this acid is oxidised only by relatively strong oxidising agents. Yet under the influence of succinoxidase, with which it no

doubt combines, an active reducing system is produced. That is what is implied by activation of hydrogen. Activated in this manner succinic acid is oxidised to fumaric acid if a suitable substance is present which it can reduce. On the other hand it seems probable that it is not sufficiently powerful as a reducing agent to reduce molecular oxygen. The evidence for this is that in the presence of traces of cyanide, the oxidation of succinic acid by oxygen is prevented even when succinoxidase is present. The succinic acid has still been converted into a reducing agent by the succinoxidase, since it will reduce methylene blue even when cyanide is present. The cyanide therefore acts on something additional that is necessary in the normal oxidation process. It has been assumed on good grounds that the cyanide poisons an oxygen activator without which the succinic acid cannot reduce oxygen. In other words, if succinic acid is to be oxidised in the tissues it has first to be converted into an active reducing agent, which it is not under normal conditions; but in addition, molecular oxygen has to be made into a more powerful oxidising agent. Under these conditions of widened oxidation-reduction potential the succinic acid will be oxidised. It seems likely that stable substances, such as saturated fatty acids, which are difficult to oxidise outside the body can be oxidised easily *in vivo* only because they can be converted into active reducing agents in the same way as succinic acid, and that with them also the oxidation-reduction potential will have to be increased still further by activation of oxygen before, even in their reducing or active form, they are able to be oxidised. In the anaerobic oxidases, the body thus possesses agents to facilitate oxidation which are not known *in vitro*, except perhaps in the form of finely divided or colloidal platinum or palladium. It is thus possible to understand how by this mechanism of extension of reduction potential, the relatively mild oxidising agents of the body are able to oxidise comparatively stable substances. There is evidence that activation of oxygen may not always be essential in addition to activation of hydrogen in order that certain metabolites may be oxidised by molecular oxygen. For instance, the oxidation of hypoxanthine by the milk oxidase is not inhibited by cyanide or carbon monoxide, which suggests at any rate that the activation of oxygen by an iron compound is not necessary for this reaction.

On the other hand it is conceivable that the oxidation of certain substrates is carried out in animal or plant tissues without the aid of anaerobic oxidases. In such substrates the hydrogen is already active, and the oxidation-reduction potential may be increased by activation

of oxygen alone. Phenols and polyphenols, especially the latter, are reducing agents, i.e., hydrogen in them is already in an active form. They undergo some oxidation in the air without the intervention of a catalyst. Only a small increase in oxidation-reduction potential is required to accelerate their rate of oxidation. This could be brought about by further activation of the hydrogen, e.g., by means of palladium or an anaerobic oxidase, or alternatively by the activation of oxygen by an aerobic oxidase. It is necessary to decide whether the enzymes which catalyse the oxidation of phenols in tissues or tissue extracts act by oxygen activation or hydrogen activation, i.e., whether they make molecular oxygen into a more active oxidising agent, or convert the phenol into a more powerful reducing agent. The failure of the enzyme-phenol combinations to reduce a dye such as methylene blue suggests that increased hydrogen activation does not take place. On the other hand, the fact that in presence of oxygen and the enzyme the phenol is rapidly oxidised indicates that the activity of the oxygen has been increased by the enzyme.

We have *in vitro* analogies to both these methods of extending the oxidation-reduction potential. Palladium in presence of oxygen oxidises quinol to quinone, and probably hydrogen peroxide is produced, but is at once decomposed (Wieland, 73, 74). Manganese acetate together with calcium glycollate also oxidises quinol in presence of oxygen to give quinone and hydrogen peroxide. The palladium converts the quinol into a more powerful reducing agent; the manganese converts the oxygen into a more powerful oxidising agent. Both increase the oxidation-reduction potential but by different methods. The products which result are the same. In this instance the production of hydrogen peroxide is thus not necessarily an indication that the *catalyst* has activated hydrogen.

Both these methods may be of use in living tissues, and a combination of them may be necessary to effect oxidation. Only a more detailed analysis of oxidation processes in the tissues will demonstrate the part played by each. There seems to be little doubt that agents which bring about both are present.

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